NATURALLY OCCURRING CYTOPLASMIC AND NUCLEAR CHANGES IN CONTINUOUSLY GROWING CULTURES OF *NEUROSPORA CRASSA*

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by

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INTRODUCTION

The many cases of non-Mendelian inheritance which have been documented during the past half century make it clear that extranuclear elements within the cell may play a role in heredity. In some of the systems which were originally used to prove the case for cytoplasmic heredity the determinants of the traits which showed extranuclear inheritance were later recognized as elements which are not normal constituents of cells, such as intracellular symbionts and parasites. That permanent, extranuclear components of normal cells may be of special significance to cellular heredity has become apparent with the recent discovery of deoxyribonucleic acid (DNA) in mitochondria and chloroplasts. The elucidation of the role of extranuclear genetic material in the physiology of the integrated cell undoubtedly will require genetic and biochemical analysis of a variety of extrachromosomal mutants. Since among the fungi Neurospora crassa is uniquely suited for the study of the genetics of the mitochondrial system, it is particularly desirable to obtain mitochondrial mutants of this organism. The lack of selective systems for obtaining such mutants has made it necessary to consider a variety of experimental methods to search for useful variants for further study.

Following the lead of Jinks (1954, 1956, 1957, 1959), who used Aspergillus to demonstrate that phenotypic changes arising during long term vegetative propagation are determined

by cytoplasmic factors, McDougall and Pittenger started a continuously growing culture of Neurospora in June of 1961 for the purpose of making an analysis of some somatic cell variations which would appear during prolonged hyphal growth. Preliminary studies suggested that some of the changes in phenotype observed in this culture were determined by extranuclear factors (McDougall and Pittenger, 1962; McDougall, 1963). The studies reported in this dissertation include not only a detailed description of the somatic variation which arose during more than four years of growth of this culture, but also genetic analyses of some of the altered phenotypes which accumulated during continuous vegetative propagation. The results will show that at least some of the somatic changes had a genetic basis, and that both nuclear and extranuclear factors contributed to the observed variation. A detailed description of the properties of two cytoplasmic mutants is included, with some biochemical evidence for the mitochondrial nature of these variants. Brief reports of some of the findings have been published previously (Bertrand and Pittenger, 1964; Bertrand, 1966).

LITERATURE REVIEW

Long Term Vegetative Propagation

Only a few observations on somatic variation during long term asexual propagation have been reported in the literature. Several phenotypic changes affecting vigor, asexual

and sexual reproductive capacity were observed in lineages of Aspergillus glaucus, A. nidulans and Penicillium cyclopium maintained either by serial transfers of hyphal tips, or by repeated subculturing through asexual spores (Jinks, 1954). Using heterokaryosis, Jinks (1954, 1956, 1957) was able to demonstrate the extranuclear origin of the determinants responsible for the differences in phenotype which arose during prolonged vegetative propagation. However, only cumulative changes in the equilibrium of normal cytoplasmic factors appeared to account for the differences, i.e. the variant phenotypes could be eliminated by selection, by heterokaryotic association with wild-type strains, and by sexual reproduction. Considering these properties, Jinks (1958, 1966) proposed that permanent losses or changes in function of cytoplasmic elements could not account for the naturally occurring cytoplasmic variation. Nevertheless, the phenotypic differences were persistent over long periods of growth in the absence of selection, and furthermore, were transmitted to successive generations of progeny derived from asexual spores (Jinks, 1958).

Prolonged hyphal propagation of Aspergillus glaucus (Jinks, 1959) and Podospora anserina (Marcou, 1961) invariably leads to an irreversible cessation of growth accompanied by death of the hyphal tips, described as "vegetative death" and "senescence," respectively. Death usually occurs after a few months of uninterrupted growth; but in a few cases

growth ceased within a few days, whereas in others it persisted for as long as two years. The morphological changes immediately preceding death showed strict extrachromosomal inheritance. Similar observations have been made with other slow growing fungi, including Podospora setosa (Rizet, 1953), Ascobolus stercorarius (Marcou, 1961), Helminthosporium victoriae (Lindberg, 1959), and Pestalozzia annulata (Chevaugeon and Digbeu, 1960). A related condition, "natural death" (nd), has been studied in Neurospora (Sheng, 1950), though in this case the responsible factor has been identified as an ultraviolet induced mutation which could be mapped in one of the linkage groups of the organism. The regularity with which "vegetative death" and related upsets were observed has led to the conclusion that indefinite vegetative propagation in fungi is impossible (Marcou, 1961).

Phenotypic modifications related to chromosomal mutations arising during prolonged vegetative propagation of fungi apparently have not been described in the literature.

Outside of the fungi, Paramecium (Sonneborn, 1954) appears to be the only organism which has been used for a systematic study on the effects of prolonged asexual propagation. The degenerative changes in vegetatively maintained clones of this protozoan follow much the same course as in fungi, but the basis for the degeneration has been traced to a progressive imbalance in the chromosomal complements of the macronucleus, rather than to alterations in the

extrachromosomal complement (Dippel, 1955; Sonneborn and Schneller, 1955a, 1955b).

Cytoplasmic Inheritance

Comprehensive discussions of extrachromosomal genetic systems in general have been published in two recent books, one by Jinks (1964), the other by Wilkie (1964). The literature describing the properties and nature of extranuclear variants of fungi has been reviewed recently by Jinks (1966), and need not be considered further in this dissertation, except as pertaining to the detection and characterization of new cytoplasmic mutants of Neurospora.

It appears that extrachromosomal mutations are detected more readily in some fungi, specifically yeast and Aspergillus (Jinks, 1966) than in most other organisms thus far examined. However, significant numbers of cytoplasmic mutants have not been observed in Neurospora. Only eight mutants of this type have been reported in the literature: five of spontaneous origin, poky (mi-1; Mitchell and Mitchell, 1952), mi-3 (Mitchell, Mitchell and Tissieres, 1953), mi-4 (Pittenger, 1956) and abn-1 and abn-2 (Garnjobst, Wilson and Tatum, 1965; Diacumakos, Garnjobst and Tatum, 1965); two, SG (Srb, 1958, 1963) and stp (McDougall and Pittenger, 1966), were induced by treatments with acriflavin and ultraviolet light, respectively; and one, AC-7 (Srb, 1963) whose origin is not known. The two mutants described by Srb (1963) are unusual in that

crosses of AC-7 with wild type, no matter how they are made, produce only wild type progeny, whereas under certain conditions SG may be transmitted to the progeny even if SG is used as the conidial parent.

Cytoplasmic variants of Neurospora have been isolated only at low frequencies after treatments with acriflavin and ultraviolet light (Srb, 1963; McDougall and Pittenger, 1966), in contrast to high frequencies of cytoplasmic petites recovered from treatments of yeast cells with the same mutagens (Ephrussi, 1953). High frequencies of induction of a variety of extrachromosomal mutants by both mutagenic agents also have been reported for Aspergillus (Jinks, 1966).

Delayed germination and slow initial growth of sexual and asexual spores are characteristic of poky (Mitchell and Mitchell, 1952) and mi-3 (Mitchell, Mitchell and Tissieres, 1953). Incapacity to function as a protoperithecial parent in crosses is a feature of mi-4 (Pittenger, 1956), abn-1 and abn-2 (Garnjobst, Wilson and Tatum, 1965), and stp (McDougall and Pittenger, 1966). At least three of the latter mutants, abn-1, abn-2 and stp, show reduced formation of conidia. When asexual spores from the four female sterile variants are plated on sorbose medium, most do not germinate, but some produce microcolonies. Asexual segregation into classes with respect to colony size has been observed in platings of conidia from homokaryons of at least one of these mutants, namely stp (McDougall and Pittenger, 1966). Segregation

into phenotypically distinct types of progeny derived from asexual spores appears to be a common characteristic among known cytoplasmic mutants of Aspergillus (Jinks, 1964).

Abnormal cytochrome systems have been found in several cytoplasmic mutants of Neurospora, including poky and mi-3 (Mitchell, Mitchell and Tissieres, 1953), AC-7 (Srb, 1963), and abn-1 and abn-2 (Garnjobst, Wilson and Tatum, 1965). The abnormalities all involve an excess of cytochrome c and a deficiency of cytochrome a. Cytochrome aberrations have not been found in the case of SG (Srb, 1963), but may be present in stp (McDougall and Pittenger, 1966). Mitochondria from poky and mi-4 are deficient with respect to succinic acid oxidase and/or cytochrome c oxidase activities (Haskins, Tissieres, Mitchell and Mitchell, 1953; Pittenger, 1956) and show altered malate dehydrogenase kinetics (Munkres and Woodward, 1966). Studies on the mitochondrial enzymes of the other mutants of Neurospora apparently have not been reported. Defects in the cytochrome system and low succinic acid oxidase and cytochrome c oxidase activities also are characteristic for the cytoplasmic petites of yeast (Ephrussi, 1953).

The only known case of synergistic interaction between two different cytoplasmic mutants has been described by Pittenger (1956). An essentially normal growth rate resulted from cytoplasmic fusions between strains of poky and mi-4, though normal growth was not permanent. Despite the

stimulation of growth, the mycelium of the heterocytosome always had mutant cytochrome systems. The synergistic action of the mutants could be explained in one of two ways: either poky and mi-4 have complementary action, or the mutant cytoplasmic constituents can cooperate to form the normal cytoplasmic constituent (Pittenger, 1956).

Jinks (1964, 1966) outlined the criteria which extrachromosomal bodies must satisfy before their role as bearers of hereditary determinants can be accepted. They must determine some cell characteristic, they must be self duplicating in the sense that, if lost, they cannot be regenerated by chromosomal material, and they must be transmitted to daughter cells during cell division. Centrioles, basal granules, plastids and mitochondria appear to satisfy these criteria. The fungi provide no evidence which implies that extrachromosomal structures other than mitochondria are involved in cytoplasmic heredity (Jinks, 1966). Mitochondrial involvement long has been suggested by the non-Mendelian inheritance of respiratory deficiencies in petites of yeast (Ephrussi, 1953), and of disorders in the electron transport system of the mi variants of Neurospora (Mitchell and Mitchell, 1952; Mitchell, Mitchell and Tissieres, 1953; Pittenger, 1956).

Labeling patterns, the content of mitochondrial phospholipids (Luck, 1963), and mitochondrial density shifts (Luck, 1965) in logarithmically growing mycelium suggest

that the mitochondria of Neurospora increase in number by growth and division of preexisting mitochondria. These mitochondria (Luck and Reich, 1964), like mitochondria from other organisms (Kalf, 1963; Corneo, Moore, Sanadi, Grossmann and Marmur, 1966), contain DNA of high molecular weight and characteristic buoyant density, and possess DNA dependent RNA-polymerase activity. The DNA is physically conserved during replication in the course of vegetative growth and asexual reproduction, and follows strict maternal transmission in interspecific crosses between Neurospora sitophila and N. crassa (Reich and Luck, 1966). Mitochondrial fractions isolated from an abn-1 strain produce phenotypic changes characteristic of abn-1 when microinjected into hyphal compartments of normal strains (Diacumakos, Garnjobst and Tatum, 1965). These results strongly support the postulate that mitochondria of Neurospora contain genetic information. There seems to be general agreement that this information is encoded in mitochondrial DNA (Gibor and Granick, 1964; Horowitz and Metzenberg, 1965; Tatum, 1965), though rigorous experimental evidence has yet to be provided.

THEORETICAL CONSIDERATIONS

At least two aspects of Neurospora make the isolation of additional cytoplasmic mutants particularly desirable: (1) the most useful and convincing insights into the mechanisms of extrachromosomal heredity have been gained from

studies performed with this organism, and (2) the fungus has several biological attributes advantageous for genetic and biochemical investigations of the nature and function of non-chromosomal genetic information in the context of the integrated cell. In addition to these two aspects, the long and extensive use of the mold for biological research has produced a wealth of information about the organism, and has created numerous laboratory techniques for its experimental manipulation; both of which should prove useful for further systematic inquiry into extranuclear genetic systems.

Prolonged hyphal propagation of fungi is purely a laboratory technique, and probably does not occur in nature where favorable environmental conditions cannot be expected to persist indefinitely. Rather, the propagation of fungi in nature seems to depend predominantly on the formation and dissemination of asexual spores. This method of perpetuation, however, does not appear to result in significant somatic variation in Neurospora. Laboratory stocks of this fungus have been maintained for many years by repeated subculturing through asexual spores, without definite indications of any cytoplasmically determined variation, but nuclear mutations occasionally result in detectable phenotypic alterations.

Neurospora crassa has a number of characteristics which are useful for the study of the effects of long-term hyphal growth. At optimum temperature, the mold normally grows at

a consistent rate on synthetic medium, often in excess of 100 mm/day. Its growth rate is ten to fifty times higher than those routinely observed for the fungi used in other growth studies. The specific growth performance of any strain undoubtedly is the product of the interaction of a number of genetical and environmental factors. If the latter are controlled carefully, changes in rate of a growing culture should be indicative of alterations in the overall physiological condition of the mycelium. These changes may or may not be hereditary.

Obviously, all genetic changes taking place during prolonged vegetative growth do not necessarily affect the rate of hyphal elongation. Since Neurospora is a coenocytic organism, many replicas of the nuclear and extranuclear genetic complements are present in a common cytoplasm. Single mutational events in either complement probably are rarely, if ever, detected, unless sufficient numbers of mutant replicas are accumulated in the mycelium. Accumulation by chance alone must be considered as extremely improbable, but there may be a few mutations which favor systematic accumulation during growth; for example, those which produce increases in the rate of division of nuclei which bear the mutation, and extrachromosomal mutations which result in an increased rate of multiplication of autonomous, or partially autonomous, cytoplasmic organelles. It is conceivable that in such cases the accumulation would be aided

by vigorous cytoplasmic streaming and frequent anastomoses between hyphae, both present in Neurospora, since these processes assure continuous and effective mixing of hyphal contents at the growing frontier of a culture.

In addition to growth rate, other properties of Neurospora are useful in following somatic variations arising during prolonged growth. Mutations often have adverse effects on the capacity of a strain to reproduce sexually or asexually. Though by far the majority of these mutations are of the chromosomal type, all cytoplasmic mutants thus far described have some characteristic abnormalities in their sexual and asexual reproductive systems. This suggests that some mutations at the chromosomal, as well as at the extrachromosomal level, can be detected through periodic evaluations of the reproductive capacity of continuously growing cultures.

The properties of Neurospora which are particularly advantageous for the unambiguous distinction between genetic determinants of chromosomal and extrachromosomal origin are heterothallism and heterokaryosis. Mating type, rather than sex, is the mechanism of autosterility, and, therefore, it is possible to obtain reciprocal crosses between two functionally hermaphroditic strains with opposite mating types, A and a. The inheritance of characters determined by chromosomal genes is not influenced by the way in which a cross is made. The transmission of a cytoplasmic character, however,

is dependent upon its determinants being carried by the protoperithecial parent. This parent is believed to furnish most, if not all, cytoplasm of the ascospores (Dodge, 1935; Sansome, 1945).

The formation of stable heterokaryons is the consequence of hyphal fusion between two different, genetically compatible, strains of the same mating type (Beadle and Conradt, 1944; Gross, 1952; Garnjobst, 1953; Holloway, 1955; Garnjobst and Wilson, 1956; Pittenger, 1964). Protoplasmic streaming assures thorough mixing of the hyphal contents (Atwood and Pittenger, 1955), resulting in the coexistence of different nuclei in a common cytoplasm. Since conidia are formed by abstrictions of specialized hyphae, they can be considered small samples of protoplasm. Random assortment of nuclei during conidia formation permits the reextraction of the component homokaryons from heterokaryons. By genetically marking the nuclei it is possible to follow each nuclear component through asexual spore production.

If a phenotypic difference associated initially with one nuclear component is transmitted to the other component during heterokaryosis, or fails to remain in association with the nucleus with which it entered the heterokaryon, then this difference must be under cytoplasmic control (Jinks, 1954). There is no unequivocal proof that nuclear fusion and recombination between nuclear genes does not occur in heterokaryons of Neurospora. Rather, the majority

of the evidence indicates that they do not take place, and that different nuclei merely share a common cytoplasm (Case and Giles, 1962; Coyle and Pittenger, 1965).

The abstriction of portions of protoplasm during conidia formation also provides a mechanism for the asexual segregation of extrachromosomal factors in homokaryons. This type of segregation is possible only if at least two distinct forms of the same factor, or possibly two different factors, are present in the same cytoplasm. Independent assortment during abstriction of conidia should result in segregation into phenotypically distinct classes of asexual progeny, or possibly into a spectrum of phenotypes. Since the nuclear background presumably is the same for all asexual progeny derived from a homokaryon, any phenotypic differences among these progeny should be determined by cytoplasmic factors. However, ideal homokaryons, i.e. cultures in which all nuclei are genetically identical, probably do not exist very long, because mutations can be expected to occur in some of the nuclei as time passes. These mutations would result in an ever increasing heterogeneity in the nuclear population of the mycelium. Segregation into distinct phenotypes also may be the consequence of the independent assortment of nuclei from a heterogeneous population during the formation of multinucleate conidia, and thus, asexual segregation in homokaryons by itself does not prove the extranuclear nature of a genetic determinant, at least not in Neurospora.

Nevertheless, persistent segregation in all progeny of several asexual generations cannot be explained by any known chromosomal or nuclear cause of segregation, and is indicative of the involvement of cytoplasmic determinants.

MATERIALS AND METHODS

Strains

The following strains of Neurospora crassa were used in this study: an adenine-requiring albino (ad-4 al-2A) selected from a series of three backcrosses of ad-4 F-4A (Woodward, Partridge and Giles, 1958) to nic-1 al-2 88a; pan-1 al-1 23A (pantothenic acid, albino); nic-1 al-2 88a (nicotinic acid, albino); nic-1 al-2 55A; standard wild type 74-OR-8-1a; pan-1 al-1 A poky, prepared from a cross of poky a (Fungal Genetics Stock Center, number 386) and pan-1 al-1 23A; and pan-1 23A stp (cytoplasmically determined stop-start growth; McDougall and Pittenger, 1966). All strains were isogenic for heterokaryon compatibility, except 74-OR-8-1a. Abbreviated designations were used as follows: ad for ad-4 al-2A, pan for pan-1 al-1 23A, nic for nic-1 al-2 55A, and 74a for 74-OR-8-1a. More complete designations were used whenever necessary.

Media and Growth Conditions

Routine growth rate determinations were made in 500 mm long growth tubes (Ryan, Beadle and Tatum, 1943) containing 30 ml of Vogel's (1956) medium with 1.5% w/v sucrose as a

carbon source, and solidified with 1.5% w/v unwashed agar. All cultures were grown at 30° C, unless specified otherwise. Crosses and conidial platings were made in Petri plates on Westergaard's medium (1947). When colonial growth was desired, sucrose was replaced by 1.0% w/v sorbose and 0.01% w/v glucose. Supplements were used at the following concentrations: adenine, 0.2 mg/ml; nicotinamide and calcium pantothenate, 0.005 mg/ml. Complete medium was prepared by adding 0.25% w/v caseamino acids and 0.25% w/v yeast extract to either Westergaard's or Vogel's medium containing the appropriate carbon source. Tests for metabolic requirements were made on Vogel's liquid medium. Nuclear ratios were calculated by the formula of Atwood and Mukai (1955). The technique described by Strickland (1960) was employed to obtain unordered tetrads. Special techniques are described in conjunction with the presentation of the experimental results.

Perpetual Hyphal Propagation

Special growth tubes designed for studying the effects of perpetual hyphal propagation have been described by McDougall and Pittenger (1962). These tubes are approximately 615 mm long and have an inside diameter of about 25 mm. Four sampling ports are spaced along the tops of the tubes, and the ends have 24/40 standard taper ground glass interchanges. Closures made of the same type of glass joints

were used for sealing ends which were not joined with other tubes. Each section was filled with 100 ml of Vogel's adenine medium. The ad-4 al-2A strain of Neurospora was innoculated at the proximal end of the first section. The distal closure of this section was removed and another section was joined to the first one before the culture reached the distal end. Once the mycelium was well established in the new section, the tubes were disjoined and sealed with sterile closures. Subcultures were prepared from masses of aerial hyphae and conidia removed from under the sampling ports before the proximal section was discarded. Repeated addition of new sections has permitted uninterrupted hyphal propagation for more than four years without need of subculturing the organism.

Detection of Mutations

The criteria used to detect cytoplasmic and nuclear alterations during continuous hyphal propagation were: (1) prominent changes in growth rate; (2) visible changes in the morphology of the fungus, such as in the abundance of aerial growth and in the production of conidia; (3) changes in the sexual and asexual reproductive capacity; and (4) changes in the morphology of colonies formed on sorbose medium.

Heterokaryons and Recovery of Homokaryons

Heterokaryons were prepared by three methods: (1) by superimposing conidia from two strains with different nutritional requirements on minimal medium, if nuclear ratios were not decisive; (2) by the technique of Pittenger and Atwood (1956), if controlled nuclear ratios were desired; and (3) by superimposing conidia from one strain on 36-hour-old mycelium of another slow growing strain, if disproportionate nuclear ratios could not be attained by the second method. The last method frequently gave heterokaryons which had a high proportion of nuclei from the slow growing component, and was found to be particularly useful when cytoplasmically determined phenotypes were transferred from one strain to another.

Heterokaryon formation was detected by growth on minimal medium. Wild-type pigmentation of the asexual spores served as an additional criterion, if the component strains carried complementing albino markers, al-1 and al-2. Homokaryons were recovered from the heterokaryons by plating samples of conidia on three kinds of media: minimal, minimal with one supplement, and minimal with the second supplement. The colony counts obtained from these three kinds of media permitted the calculation of nuclear ratios and an approximation of the number of colonies necessary for the recovery of at least some homokaryons. Colonies were then transferred from plates with the appropriate medium to test tubes

containing medium supplemented with the metabolite required by the desired homokaryon. Following conidiation each single colony isolate was tested for growth on liquid minimal medium. Those which did not grow were assumed to be homokaryons. Since all heterokaryon-compatible strains were albino, albinism served as an additional criterion for homokaryosis, provided the components were marked with non-allelic genes determining albinism.

Enzyme Studies

Crude Enzyme Preparations. Mycelium from logarithmically growing cultures was harvested on filter paper in a Buchner funnel. The pad was washed three times with about 20 volumes of distilled, deionized water, and suspended in 15 ml of cold 0.44 M sucrose-0.001 M EDTA (EDTA-sucrose). The mycelium was homogenized for 30 sec at low speed with a Virtis homogenizer. Subsequently the homogenate was shaken for 30 min with 20 ml of glass beads (average diameter: 1.0 mm) and three large glass marbles in a 125 ml Erlenmeyer flask. The disrupted mycelium was washed from the beads by adding small amounts of EDTA-sucrose and decanting excess liquid into a centrifuge tube. Cell walls and nuclei were removed by centrifuging twice for 10 min in a clinical centrifuge set at full speed, and once for 10 min at 3000 X g in an International centrifuge. The remaining supernatant was centrifuged at 13,000 X g for 30 min. The mitochondrial

sediment was washed by resuspending the pellet in 25 ml of EDTA-sucrose and recentrifuging at 13,000 X g. The final pellet was resuspended in 1.0 ml EDTA-sucrose, and was used in this form as crude enzyme preparation for determinations of cytochrome c oxidase and cytochrome c reductase activities. Protein was determined by the method of Lowry et al. (1951).

Cytochrome c Oxidase. The assay for cytochrome c oxidase activity in crude preparations of mitochondria was performed by a method adapted from that described by Smith (1955). The reaction mixture contained 2.7 ml of 0.01 M potassium phosphate buffer, pH 7.4; 0.090 mM of reduced cytochrome c (Sigma; horse heart, type III), and 0.025 mg of enzyme protein. The final volume of the mixture was 3.0 ml. The reaction was followed by recording the decrease in absorbance at 550 millimicrons by means of a Beckman DK-2 recording spectrophotometer. Complete oxidation of cytochrome c was attained by adding a drop of a saturated solution of potassium ferricyanide to the reaction mixture. Cytochrome c was reduced by adding a few small crystals of sodium dithionite to a solution of cytochrome; excess reducing agent was removed by bubbling air through the solution. Enzyme activity was related to the first order velocity constant for the oxidation of cytochrome c at 37° C.

Cytochrome c Reductase. Cytochrome c reductase activity in crude preparations of mitochondria was determined by a modification of the procedure described by Stotz (1955).

The reaction mixture contained 2.5 ml of 0.01 M potassium phosphate buffer, pH 7.4; 0.090 mM of oxidized cytochrome c, 0.1 ml of 0.02 M neutralized KCN, 0.1 ml of 0.2 M sodium succinate, and 0.025 mg of enzyme protein. The final volume was 3.0 ml. The rate of reduction of cytochrome c was followed by recording the increase in absorbance at 550 millimicrons. A unit of activity was defined as the amount of enzyme which produces a change of 1.0 in optical density per minute at 37° C.

RESULTS

Growth Rates and Naturally Occurring Changes

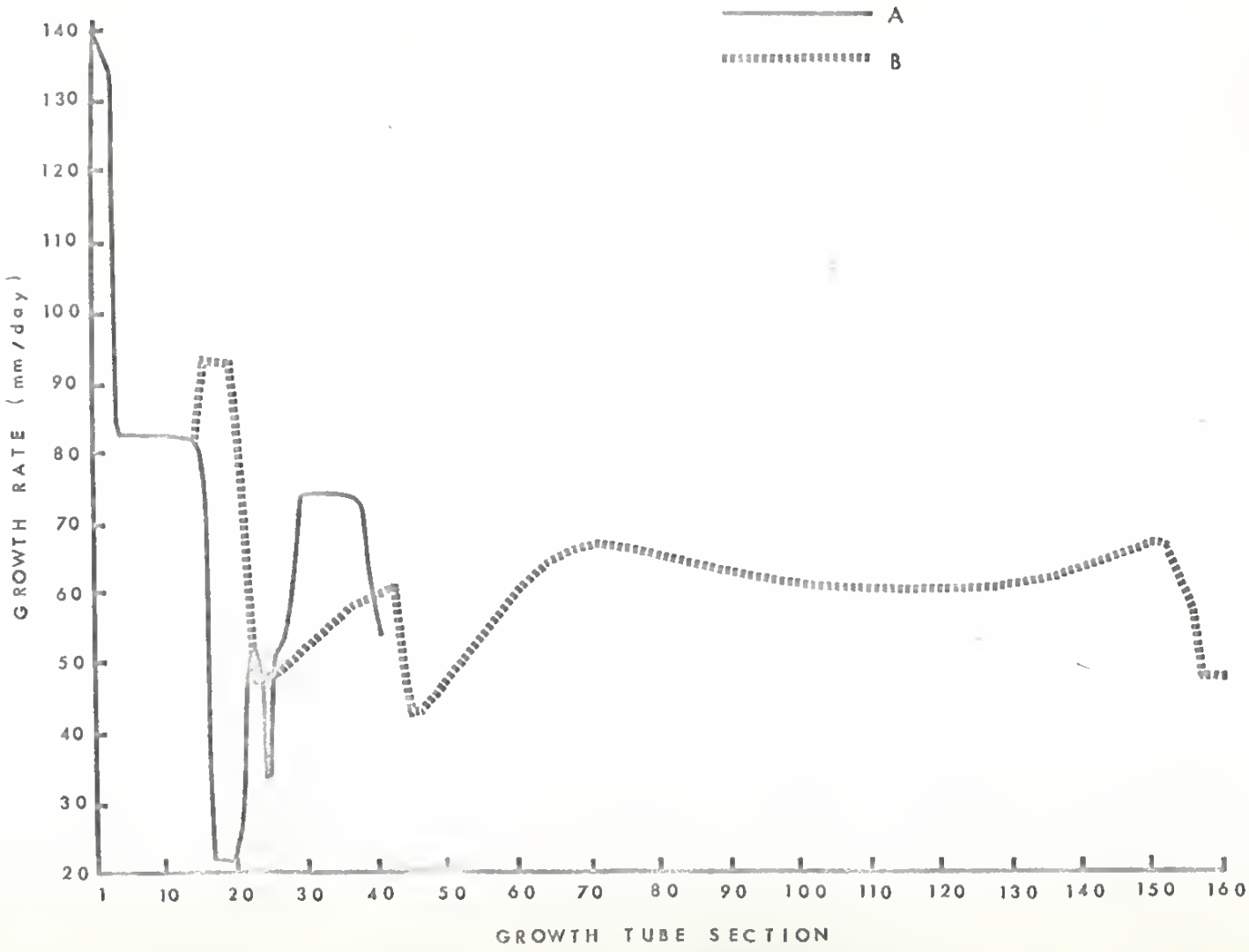
Some of the changes in growth and morphology which occurred during prolonged hyphal growth of the ad culture of Neurospora used for this study have been described previously in abbreviated form (McDougall and Pittenger, 1962; McDougall, 1963; Bertrand, 1966). The strain was inoculated on June 9, 1961 onto Vogel's adenine medium in the first section of a continuous growth tube. This culture was kept at 30° C until growth was discontinued. The advancement of the hyphal tips was marked daily on the outside of the tube, and measurements were made after the mycelium had advanced well into the next tube. Subcultures were prepared and stored as described in Materials and Methods.

A branch tube was started from the proximal end of section A16 and parallel lines of growth were maintained

subsequently. For the sake of clarity, the main line of growth has been called A-growth tube, and its sections were numbered from A1 to A41. The branch growth tube has been designated the B-growth tube, and its sections were numbered from B16 to B160. The respective cultures will be referred to as the A- and B-cultures. The average growth rates of the culture for each section of both growth tubes have been plotted in Figure 1. Because of the inconsistent daily rates observed in sections between A23 and A41, the highest 24-hour performance has been used for these tubes, rather than the average of all daily rates. This procedure was adopted because it was felt that in this manner the growth rate would be a more accurate expression of the potential rate of the culture during growth in that segment of the A-growth tube.

Description of Growth in the A-growth Tube. The ad culture grew at 137 mm/day through the first 1200 mm of the A-growth tube. It rate declined progressively to an average of 34 mm/day within the next 650 mm of growth. Subject to minor variations, the latter rate was maintained over a distance of 7400 mm in sections A4 through A15. After a total of 9640 mm of growth in 101 days, there was another gradual decline in the growth rate of the culture, from 84 mm/day in section A15 to 24 mm/day in the distal half of section A16. Growth was erratic in sections A17 through A20, with daily rates ranging from as low as 9 mm/day to as high

Figure 1. Growth rates of the *ad-4 al-2 A* strain of Neurospora during continuous hyphal elongation in growth tubes A and B. Rates during slow growth associated with stopping, and periods of inactive hyphal elongation were excluded from the calculations. Because of erratic growth, best 24-hr performances instead of average rates were plotted for sections A23 through A41 (see text for full explanation). The letters in the graph indicate the corresponding growth tubes.


A
B
GROWTH RATE (mm/day)
140
130
120
110
100
90
80
70
60
50
40
30
20
1
10
20
30
40
50
60
70
80
90
100
110
120
130
140
150
160
GROWTH TUBE SECTION

as 41 mm/day, with an average of 19 mm/day. A gradual improvement was noticed in section A21, until the frontier of the culture advanced at a steady 48 mm/day over a distance of about 800 mm in section A22 and part of A23. A third progressive decline began in the distal half of section A23. This time hyphal growth stopped completely six days after the onset of the decline. By then the culture had grown a total of nearly 14,200 mm in 305 days.

Beginning with the stop in section A23, the ad culture of the A-growth tube grew in highly irregular cycles consisting of a period of active hyphal elongation followed by a stop. For example, growth resumed in section A23 at a rate of 38 mm/day after 8 days of absence of measurable hyphal elongation. However, three days later the culture ceased growing again, this time for 10 days in section A24. This stop was in turn followed by 31 days of active and 15 days of inactive growth.

The stop-start growth pattern was maintained until the culture was discarded in section A41, about 1065 days after stopping was first noted in section A23. The periods of active growth ranged from 1 day to 31 days, during which the hyphal tips advanced from 5 mm to 1,050 mm. The periods of total absence of measurable growth ranged from 1 day to over 3 months. The highest daily growth rates during individual periods of active growth ranged from 4 mm/day to 91 mm/day. All in all, the culture stopped growing 48 times for a total

of 780 days, and active hyphal elongation was observed during only 285 days after the culture had first stopped in section A23.

Other Changes in the A-culture. The decrease in growth rate in section A16 was accompanied by several changes in characteristics of the ad strain of Neurospora originally used to start continuous hyphal elongation. Whereas abundant formation of conidia appeared in subcultures corresponding to all sections prior to A16, the production of asexual spores was greatly reduced in samples corresponding to sections A16 and A17. No conidia could be detected microscopically in samples of mycelium extracted from sections between A31 and A41. However, abundant formation of conidia did take place in a fraction of the subcultures which were grown on agar slants at 30° prior to storage at 4°, but no asexual spores were formed when these conidiating subcultures were grown in 600 mm growth tubes, or even when the main culture was subcultured directly in 600 mm tubes. The reason for the formation of conidia on slants, and not in growth tubes, is not known, but it appears that it was purely environmental.

The A-culture also lost the capacity to function as a protoperithecial parent. This loss was one of the changes which accompanied the decline in growth rate in section A16. Functional protoperithecia were formed by subcultures of all sections prior to A16, as concluded from the appearance of

fertilized perithecia after spreading conidia from strains with a mating type over 5- or 6-day-old ad A mycelium grown on Westergaard's medium. No fertilization was noticed when subcultures from section A16, and subsequent sections, were tested.

The appearance of a brown pigment in aging mycelium and a brown discoloration of the culture medium were other changes which became apparent after growth had slowed in section A16. The discoloration of the medium was particularly noticeable in regions of stopped hyphal tips. A progressive deterioration of aging mycelium, which has been called "lysis" (McDougall, 1963), was also noticed in regions where abundant aerial hyphae had formed. The probable genetic basis of all these changes will be discussed in later sections of this paper.

Description of Growth in the B-growth Tube. It was of interest to know whether the agent(s) which caused the drastic changes in growth morphology, and reproduction of the ad strain in section A16 was already present, but unexpressed, in the proximal end of this section. A new section was connected to the proximal end of section A16, in addition to the usual procedure of connecting a fresh section to the distal end. A second continuously growing culture was started in this manner, and the corresponding growth tube has been designated the B-growth tube.

The culture of Neurospora which was started from the proximal end of section A16 began to grow at an average of 92 mm/day. This rate was considerably higher than the 63 mm/day observed in the proximal half of A16; in fact, it was even above the 84 mm/day observed for A15 and previous sections. This improved rate was maintained during the first 2460 mm of growth in sections B16 through B19. A gradual reduction occurred within the subsequent 2000 mm of hyphal elongation, reaching a low 48 mm/day in the proximal end of B23. After nearly 4500 mm of elongation in 55 days, growth ceased abrubtly in B23, but resumed 11 days later. Another 27-day cessation occurred after the culture had progressed 145 mm in 4 days.

No further stopping was observed during the subsequent 519 days in which the B-culture grew from A23 to A70, a total of 28,740 mm. Some changes in rate, nevertheless, occurred during this period (Figure 1). There was a slow increase from 43 mm/day in the distal one-third of B23 to 63 mm/day in B36 through B39. A gradual decline followed, to an average low of 42 mm/day between B46 and B48. The rate again increased slowly to 68 mm/day in B65 in about 10,000 mm of growth. The B-culture continued to grow at rates averaging 60 mm/day to 67 mm/day up to B151, except for short gradual decreases in the 3 or 4 days prior to total stopping.

The B-culture grew in a stop-and-go fashion in sections between B70 and B75, inclusive. However, contrary to the

predominantly long periods of inactive growth observed in the A-culture, the B-culture never stopped longer than four days during its growth from B69 to B160. Growth ceased twice for 2 days in B70; once for 2 days in B71; once for 2 days, and once for 4 days in B72; once for 3 days in B73; and once for 3 days, and again for 2 days in B75. The distances between successive stops ranged from 254 mm to 1020 mm, corresponding to the shortest (4 days) and longest (15 days) periods of active growth, respectively.

The 3490 mm of stop-start growth from B70 through B75 were followed by about 6300 mm of uninterrupted hyphal elongation. Another series of stops began in B86, and ended after the culture had progressed 1900 mm to B89. The durations of the 5 stops were, in order of occurrence, 2 (B86), 2, 1, 1 (B88), and 3 (B89) days, respectively. Although subnormal growth rates were apparent in the vicinity of three closely spaced stops in B88, the culture continued to grow at a rate comparable to that observed prior to the series of stops.

The pattern of growth described for the segment of the B-growth tube between B75 and B89, i.e. a long period of uninterrupted hyphal elongation followed by a series of stops, was repeated twice more. After stopping in B89, the culture grew 9570 mm in 158 days without stopping. Four stops occurred within 650 mm in sections B104 and B105. Another period of continuous growth followed, during which

the frontier of the mycelium advanced 6690 mm in 108 days. Only a single one-day stop occurred in B116. No further stopping was observed during the remaining 27,000 mm and 461 days of growth until the culture was discontinued in B160.

There was only one major change in growth rate between sections B100 and B160. A marked decrease took place between B151 and B157, from 68 mm/day to 50 mm/day. Some fluctuations in the daily rates were observed during the reduction, such that the transition was not altogether as gradual as it appears in Figure 1. This decrease, and the one which appeared in the same growth tube between B19 and B23 were of particular interest, because both occurred simultaneously with remarkable alterations in the morphology of the ad culture.

Other Changes in the B-culture. It has been mentioned previously that the growth rate of the ad culture of the B-growth tube declined from 84 mm/day to 48 mm/day in the segment comprising sections B19 through B23. This decrease coincided at least in part with significant alterations in the morphology and reproductive properties of the culture. Subcultures from B16 and B17 showed abundant aerial growth, rich formation of conidia and functioned well as protoperithecial parents in crosses. By the time growth had reached section B23, conidia were formed only sparsely, and aerial growth was rather limited. Furthermore, the culture had lost the capacity to form functional protoperithecia.

Irregularly shaped "lysed" areas appeared in 2- to 3-week-old mycelium. "Lysis" started in discrete areas throughout the length of growth tubes, and progressively encompassed most of the surface of the mycelium.

The continuously growing culture of the B-growth tube retained this altered phenotype during about 3 years and 9 months of vegetative propagation. Because of its distinctiveness, the new phenotype has been designated variant-1 (*var-1* genotype).

A drastic modification of the *ad var-1* strain occurred at the end of the decrease in growth rate in sections B151 through B157. The culture turned aconidial at the end of B157, and aerial growth had disappeared at the beginning of B158. Excessive branching of the growing hyphal tips was noticed at this time. The genetic basis for the phenotypic changes observed during long term growth will be presented in a later section of this dissertation.

The Stopper Phenotypes

As described previously, recurrent cessation of growth was observed in both growth tubes. However, the patterns of stopping and starting of the A- and B-cultures were remarkably different. Frequent long-lasting stops and short periods of growth occurred in the A-culture, whereas active hyphal elongation in the B-culture was interrupted by only a few series of relatively short stops. These differences

are illustrated in Figure 2, where distances of growth in segments of the A- and B-growth tubes have been plotted against time. The proximal ends of sections A31 and B84 were chosen as starting points. Whereas the distance vs. time plot for the A-culture is characteristic for all growth between sections A23 and A41, that for the B-culture represents only a series of stops.

The stop-start growth phenotypes of the A- and B-cultures have been called stopper-A (stp-A genotype) and stopper-B (stp-B genotype), respectively. This designation was chosen because the properties of these growth variants are reminiscent of the characteristics of the ultraviolet induced stp mutant described by McDougall and Pittenger (1966).

Description of the Stopper-A Phenotype. Subject to slight variations, the following growth rates were determined for three control cultures: ad-4 al-2A, 83 mm/day; pan-1 al-1 23A, 104 mm/day; nic-1 al-2 55A, 115 mm/day. Subcultures of the ad stp-A strain from sections A23 through A41 usually showed a pattern of slow growth and stopping dur[illegible] two weeks of growth in 500 mm growth tubes (Figure 3). This period of abnormal growth usually was followed by 4 to 8 days of improved performance, the end of which was marked by a gradual decline in rate over a period of 3 to 6 days, and stopping. Some subcultures resumed growth after 10 to 90 days, but only a few grew

Figure 2. Characteristic growth patterns associated with ad-4 al-2 cultures having stopper-A and stopper-B phenotypes. The numbers on the growth curves indicate the number of days during which the cultures did not grow.

A - ad-4 al-2A stp-A in sections A31 through A34

B - ad-4 al-2 var-1A stp-B during a series of stops in sections B84 through B89

(See text for full explanation)

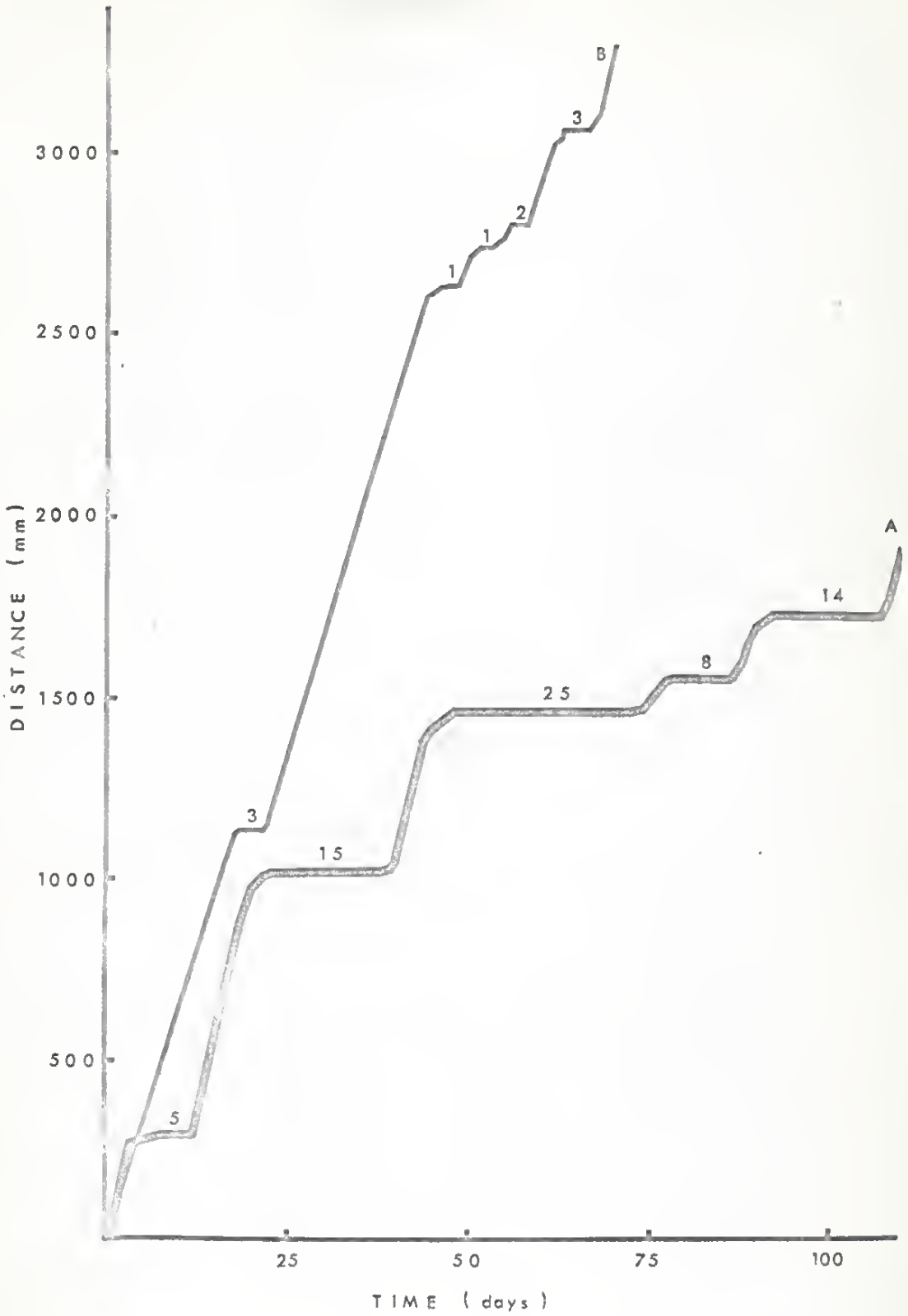
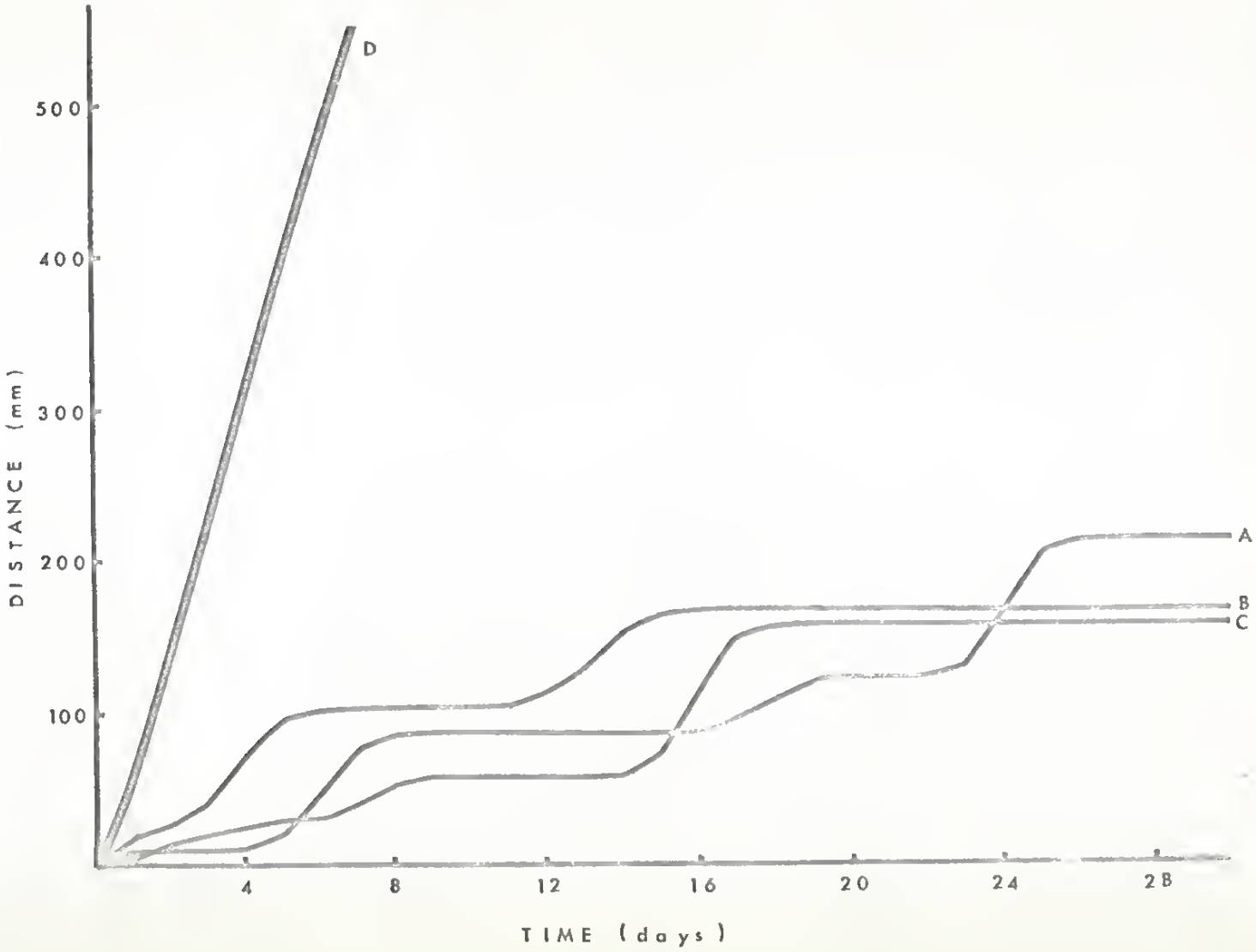

B
3
2
1
1
3
A
14
8
25
15
5
3000
2500
2000
1500
1000
500
DISTANCE (mm)
25
50
75
100
TIME (days)

Figure 3. Characteristic growth curves of stopper-A cultures.

A and B - Cultures of *ad stp-A* started with mass hyphal innoculi from section A34.

C - A single colony isolate of *ad stp-A* obtained from a plating of conidia from a subculture of section A34.

D - Control *ad*.


D
500
400
300
200
100
DISTANCE (mm)
A
B
C
4
8
12
16
20
24
28
TIME (days)

through the entire length of the growth tube before the medium dried out excessively. In addition to their erratic growth, stopper-A cultures produced no conidia in growth tubes, but asexual spores were formed by some subcultures when grown on agar slants. The conidia which were formed could not be distinguished cytologically from those formed by wild-type cultures. Platings of stopper-A conidia revealed only about 1.0 per cent germination, compared to 80 per cent or better germination for normal controls. Most of the germinated conidia did not grow beyond the germling stage (Pateman, 1960), i.e. the macroconidia formed microscopically discernible hyphae, but did not reach visible-colony status in 8 to 10 days of incubation at 30°. Only about 0.1 per cent of the conidia gave rise to visible colonies, ranging in size from micro-colonies to about half the size of control colonies. Only the most vigorous of these colonies grew significantly when transferred to growth tubes or agar slants. The growth pattern of such single conidia isolates was similar to that described for mass hyphal innoculi (Figure 3).

The viability of germlings was determined from their capacity to form heterokaryons with a normally growing strain. A heavy suspension of conidia from a normal pan strain was incubated with shaking in liquid Vogel's pantothenate medium until germination began after about 4 hours. Droplets of this suspension were superimposed on five-day-

old ad germlings, and excess liquid was allowed to evaporate. These mixtures were then transferred to agar slants of Vogel's minimal medium. A control was set up by transferring germlings only to slants of adenine medium. Since the ad and the pan strains were marked with non-allelic albino markers, wild-type pigmentation, in addition to growth on minimal medium, provided visible evidence for the formation of heterokaryons. Of a total of 210 mixtures, 56 grew and formed heterokaryons. Only 5 out of 210 controls grew appreciably. These results indicate that about one fourth of the germlings were capable of forming heterokaryons, even though they were not capable of homokaryotic growth.

Platings of conidia from 43 of the above 56 heterokaryons showed segregation for colony size similar to that observed for the ad stp-A component. However, during the first two or three days in growth tubes, the same heterokaryons grew considerably more vigorously than single colony isolates from stopper-A subcultures of the A-growth tube. This effect of heterokaryotic rejuvenation disappeared completely when the heterokaryons were subcultured once or twice before being tested in growth tubes. The expression of the stopper-A phenotype in heterokaryons with a normal strain suggests that the stp-A mutation is either dominant to its wild-type counterpart, or that the determinants for normal growth are eliminated from the heterokaryotic mycelium.

Growth of stopper-A cultures resumed 3 mm to 140 mm behind the stopped hyphal tips. This distance appeared to be related to the duration of the absence of growth, i.e. growth resumed closer to the stopped tips in the case of short periods of stopping than in the case of long periods of stopping. It was also noted that when stopping did occur it involved all hyphal ends of the culture, even though the frontier of the mycelium was spread over surfaces as wide as the diameter of a 100 mm Petri dish. These observations suggested that stopping was the consequence of, or resulted in, the incapacity of hyphal tips to support further growth. Evidence favoring this notion could be obtained by growing ad stp-A cultures in Petri plates containing solidified Vogel's adenine medium. As soon as such cultures stopped growing, 2 x 2 mm blocks of agar were cut at the edge of the mycelium, and the hyphal tips were transferred to slants of the same medium. None of 86 such transfers grew. Tips from a growing control ad culture were subjected to the same treatment, and in this case all of 60 transfers grew normally. When 2 x 2 mm blocks of mycelium were cut 20 to 40 mm behind the stopped frontier, 37 out of 46 transfers began to grow without appreciable delay.

Using a technique similar to that used for germlings, it could be shown that the capacity to grow apparently cannot be restored in stopped hyphal tips by heterokaryotic rejuvenation. None of 58 blocks of stopped ad hyphal tips

and normal pregerminated pan conidia showed any growth even 30 days after their transfer to slants. When tips from a normally growing ad culture were treated in the same manner, all transfers grew and formed visible heterokaryons. Similarly, 80 per cent of the blocks cut from areas 20 mm to 40 mm behind the stopped frontier formed heterokaryons. Failure to grow after transfer to slants and failure of "rescue" through heterokaryosis have been taken as presumptive evidence that stopped hyphal tips of stopper-A cultures are incapable of further growth, and that both components, stopper and normal, must have the capacity to grow in order to form a viable heterokaryon.

Behavior of the Stopper-A Phenotype in Crosses. It has not been possible yet to isolate a stopper-A strain which functions as a protoperithecial parent in crosses. Among 120 random viable ascospores obtained from a cross in which nic-1 al-2 88 a was used as the protoperithecial parent and a stopper-A subculture of section A36 as the conidial parent, none had the characteristics associated with the stopper-A phenotype. All progeny grew normally, showed abundant formation of conidia, and formed functional protoperithecia. The viability of the conidia from all progeny was normal, and no asexual segregation for colony size could be found on sorbose medium. The failure of the stopper-A phenotype to be transmitted in a cross in which the stopper strain is used as the conidial parent is consistent with the

interpretation that its determinants are of extrachromosomal nature.

Heterokaryotic Transmission of the Stopper-A Phenotype. McDougall (1963) has presented preliminary evidence that the stopper-A phenotype can be transferred from a stopper strain to normally growing strains by means of heterokaryosis. Heterokaryotic transmission of the phenotype could be shown conclusively by means of two heterokaryons between an ad subculture of section A34 and a normally growing pan strain. Heterokaryons 1 and 2 contained low and high proportions of pan nuclei, respectively. In both cases the stopper-A phenotype was contributed exclusively by the ad component. Both heterokaryons and their homokaryotic components were grown through 500 mm growth tubes, and single conidia isolates were prepared from the distal end of each tube. Though the growth rates of both heterokaryons fluctuated from day to day, only heterokaryon 1 stopped.

An examination of the growth patterns of 47 single colony isolates from heterokaryons 1 and 2 showed clearly that stopping failed to remain in exclusive association with the ad nucleus with which it had been introduced into the combinations. Among 42 homokaryotic isolates, 10 were ad and stopped, 6 were ad and grew normally, 14 were pan and stopped, and 12 were pan and grew normally. The remaining five isolates were heterokaryons, 3 normally growing and 2 with stopper-A growth. None of 30 isolates from the control

pan homokaryon stopped, or gave even the faintest indication of aberrant growth. Single colony isolates from the ad stp-A control homokaryon could not be obtained from the growth tube, because the culture did not grow sufficiently to reach the distal end of the 500 mm tube. Instead, 10 single colony isolates were prepared from the test-tube subculture of A34. All had growth patterns characteristic of stopper-A cultures.

Various degrees of plating efficiency, ranging from 1 per cent to 65 per cent germination of conidia, were obtained for the reextracted ad and pan homokaryons. Segregation for colony size was present in all isolates recovered from heterokaryons 1 and 2, regardless of normal or abnormal growth, and low or high plating efficiency. Normal growth and high plating efficiency in homokaryons obtained from the two heterokaryons was lost if the isolates were subcultured repeatedly, such that all isolates eventually assumed the stopper-A phenotype. Conidia formation in growth tubes disappeared after two or three transfers, and cultures which formerly grew normally began to grow in the erratic manner which is characteristic of stopper-A strains. Subculturing had no adverse effects on the normal growth, high plating efficiency, and the large, uniform size of colonies of pan control isolates.

Strains of pan which acquired the stop-start growth behavior by heterokaryotic association with an ad stp-A culture, also acquired the capacity to transmit the phenotype

to other heterokaryon-positive strains with normal growth. Four pan-1 al-1 stp-A isolates from heterokaryons 1 and 2 were put in heterokaryons with a normal nic-1 al-2 strain. A small number of nic homokaryons were subsequently recovered from each heterokaryon and tested in growth tubes. More than half of the nic isolates from each heterokaryon stopped, giving a total of 38 stopping isolates out of 55 recovered nic homokaryons. Control nic homokaryons were obtained from a heterokaryon between a normal pan and the normal nic strain, and also as single colony isolates from the normal nic homokaryon. Neither stopping nor any other indication of aberrant growth was found in more than thirty control isolates from either source.

In summary, heterokaryotic transmission of the stopper-A phenotype, like its failure to be transmitted from the conidial parent to ascospore progeny, supports the extranuclear origin of its genetic determinants.

Description of the Stopper-B Phenotype. Stopping and starting of the ad culture in the B-growth tube differed from the stop-start growth of the A-culture in that stops were of relatively short duration, and tended to occur in series between long periods of uninterrupted growth. However, permanent changes in the mycelium of the B-culture resulted in an ad-4 al-2 strain with altered morphology (see: Other Changes in the B-Culture), such that differences in growth pattern by themselves could not be considered

indicative of differences between the genetic determinants of the stopper-A and the stopper-B phenotypes, particularly since the B-culture was initiated from the proximal end of section A16 of the A-growth tube. Other evidence favoring that the two stopper phenotypes are determined by cytoplasmic factors which are qualitatively different will be presented in connection with the description and genetic analysis of the stopper-B phenotype.

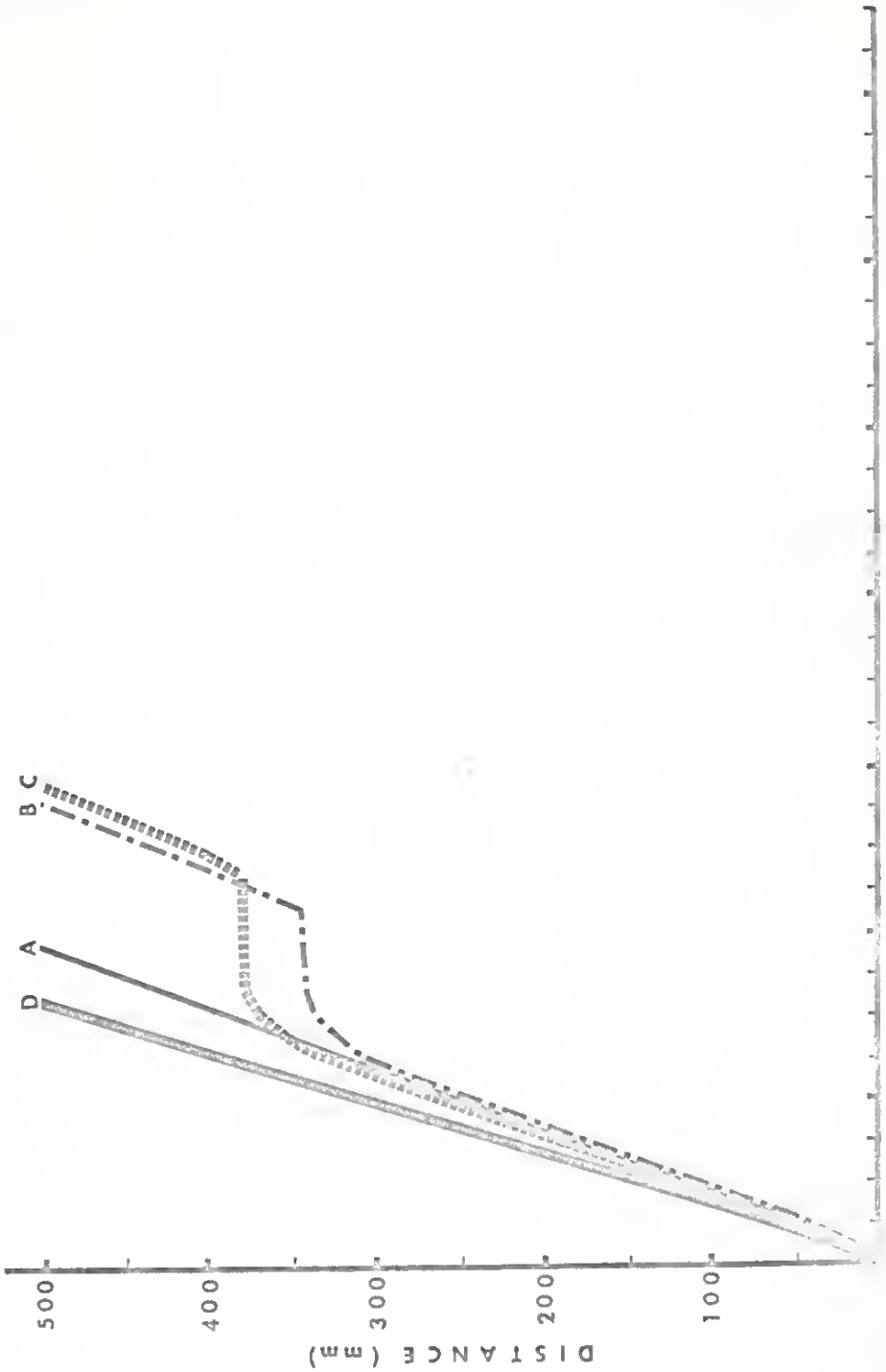
Single conidia isolates from the B-culture were prepared either from conidia of subcultures from various sections of the growth tube, or from conidia which were obtained directly from the continuously growing culture itself. Most of the isolates attained maximal growth rates during the second day after innoculation in growth tubes (Figure 4). After the fifth to tenth day of active hyphal elongation, the rates of a variable fraction of the isolates decreased gradually over a period of about three days, after which hyphal elongation came to a complete stop. Total absence of hyphal elongation usually lasted only from one to three days, before hyphal growth resumed at a comparatively normal rate. In some cases another cycle of stopping was observed within a week after the first one. Stopper-B single conidia isolates had a considerably more uniform growth pattern than stopper-A single conidia progeny, as can be seen by comparing the growth curves in Figures 3 and 4.

Figure 4. Typical growth curves of single conidia isolates prepared from the continuously growing *ad var-1* culture of the B-growth tube.

A - Non-stopping *ad var-1* isolate

B and C - *ad var-1 stp-B*

D - *ad var-1*$^{+}$ control


B,C
A
D
500
400
300
200
100
DISTANCE (mm)
4
8
12
16
20
24
28
TIME (days)

One of the characteristics of the stopper-B phenotype was its unpredictable expression. For example, when the same isolate was grown in replicate growth tubes, stopping occurred in only a fraction of the growth lines. Since only some single conidia isolates from various cultures stopped in Ryan tubes, it was hoped that non-stopping ad cultures could be selected among the asexual progeny of samples with low proportion of stopper isolates. However, these attempts were abandoned when it was found that the absence of stopping in Ryan tubes did not indicate that the factor, or factors, responsible for stop-start growth were not present in the culture. Several individual isolates gave no indication of stopping in 10 replica growth tubes, but segregation for stopping was found among the single conidia progeny of the same isolates. For example, stopping among the single conidia progeny derived from 14 cultures, which showed no stopping when each was tested in ten replica growth tubes, was compared with stopping among the single conidia progeny from 13 cultures, each of which stopped once or more times in at least five out of ten replica growth tubes (Table 1). Stopping occurred in 23 per cent of the single conidia isolates derived from cultures which had not stopped in any one of ten growth tubes, compared to stopping in 27 per cent of the isolates from cultures which had stopped in half or more of the replica tubes. There was no stopping among the conidial progeny from 5 of the 27 cultures, but only 3 of

Table 1. A comparison of stopping among single conidia isolates derived from: (1) 14 cultures which showed no stopping when each was tested in ten replica growth tubes, and (2) 13 cultures which stopped in five or more of ten replica growth tubes. All 27 cultures were obtained as single conidia isolates from the B-culture.

Cultures that stopped in none of 10 growth tubes			Cultures that stopped in five or more of 10 growth tubes		
Culture	No. of isolates tested	No. of isolates stopped	Culture	No. of isolates tested	No. of isolates stopped
*B79-3-13	10	2	B79-3-18	10	5
B84-4-303	20	2	B84-4-304	20	1
B104-2-9	20	2	B84-4-311	13	1
B104-2-11	20	4	B82-2-341	15	6
B82-2-346	15	12	B82-2-342	15	1
B82-2-347	15	2	B82-2-343	15	0
B82-2-353	15	4	B82-2-345	15	8
B86-2-407	15	0	B82-2-348	15	11
B86-2-408	15	4	B82-2-349	15	2
B86-2-409	15	0	B82-2-350	15	0
B86-2-410	15	4	B82-2-351	15	5
B86-2-411	15	6	B82-2-352	15	8
B86-2-412	15	8	B82-2-355	15	4
B86-2-413	15	0			
Totals	220	50		193	52
Per cent	100	23		100	27

*Culture code: B79-3-13 indicates that the culture was single conidia isolate number 13 from sampling port 3 of section B79. Similar information is contained in the code numbers of all other cultures given in this and other tables.

the 5 did not stop in replica growth tubes. One random single conidia isolate was selected from each of these 5 cultures, and 20 daughter colonies from each of these random isolates were in turn tested in growth tubes. Stopping was observed in at least one of each set of 20 asexual progeny, and occurred in 21 per cent of all growth tubes (Table 2).

Table 2. Stopping among single conidia progeny prepared from one random isolate of each of five *ad* cultures which showed no stopping among single conidia isolates (Table 1). The original cultures were derived as single conidia isolates from the continuously growing *ad* culture of the B-growth tube.

Culture from which random isolate was derived	Number of single conidia isolates	
	Tested in growth tubes	Stopped in growth tubes
B86-2-407	20	8
B86-2-409	20	1
B86-2-413	20	3
B82-2-343	20	6
B82-2-350	20	3
Totals	100	21

Enrichment of the medium with yeast extract and/or caseamino acids, and incubation at different temperatures--25°, 30°, and 35°--did not affect significantly the relative number or duration of stops in replica growth of the same *ad stp-B* cultures. In contrast to the stopper-A phenotype, the stopper-B phenotype did not appear to influence the viability of asexual spores. For example, the plating efficiency of conidia from the 27 cultures given in Table 1

ranged from 73 per cent to 94 per cent, with an average of 83 per cent. Germination of conidia from a control ad culture ranged from 79 per cent to 85 per cent, with an average of 82 per cent for five independent platings.

Germlings, which are characteristic of stopper-A cultures, were found only rarely in platings of conidia from strains with the stopper-B phenotype, though there was considerable heterogeneity in the size of five- to seven-day-old colonies formed on sorbose medium. Nevertheless, stopping of single colony isolates derived from stopper-B cultures could not be predicted from colony size. A total of 106 six-day-old colonies with diameters greater than 1.5 mm, and 70 colonies with diameters smaller than 0.5 mm were transferred from Petri dishes to growth tubes. Stopping occurred in 29 per cent and 31 per cent of the growth tubes innoculated with large and small colonies, respectively. There were no obvious differences in the morphology or growth rate between cultures started with the two types of colonies.

Growth and heterokaryotic "rescue" experiments indicated that the hyphal tips of stopped stopper-B cultures are not capable of further growth on their own, a characteristic which also was found to be associated with the stopped frontier of stopper-A cultures. However, growth in stopper-B strains resumed at, or very near to, the stopped tips, unlike resumption some distance behind the tips observed for

stopper-A cultures.

Although the above description of the stopper-B phenotype applies specifically to the ad strain of Neurospora which was growing between sections B25 and B150, only minor deviations were noticed when the phenotype was introduced into strains with a different genotype. Whereas the phenotype did not appear to influence the formation of conidia in ad cultures derived from the B-growth tube, conidiation was reduced when the phenotype was introduced into pan-1 al-1 and nic-1 al-2 strains, but there was no reduction in the viability of the conidia which were formed. Another variation in the expression of the stopper-B phenotype was found in the formation of a brown pigment in aged mycelium of pan and nic cultures, but not in ad cultures derived from the B-growth tube.

The accumulation of a self-intoxicating substance during growth was considered as one possible explanation for stopping. Elimination of this substance by diffusion or decay would account for resumption of growth. To investigate this possibility, several stopper-B cultures were grown in tubes which were about 1000 mm long and had a diameter of about 25 mm. Each culture was grown in several replica tubes containing 40 ml, 80 ml, and 150 ml of medium. There was no significant difference in the frequency and duration of stops between cultures grown on different amounts of medium. Comparable observations were made with stopper-A cultures.

This result suggests that elimination by diffusion of a self-intoxicating substance is not involved in stop-start growth of the two stopper variants, if such a substance is produced at all. These experiments do not eliminate an unstable, non-diffusible factor.

Behavior of the Stopper-B Phenotype in Crosses. Similar to the stopper (McDougall and Pittenger, 1966) and stopper-A variants of Neurospora, strains with the stopper-B phenotype could not be crossed as protoperithecial parents. Twenty-nine complete asci and 75 random spores were obtained from three crosses in which 74a and ad stp-B were used as the protoperithecial and conidial parents, respectively. No stopping was found among the ascospore progeny. The segregation of nuclear factors in these crosses is described in a later section dealing with the genetic basis of changes in growth rate during long term hyphal propagation. The absence of stopping among ascospore progeny from crosses in which the stopper-B strains were used as conidial parents, together with the unpredictable expression of stopping among first, second, and third generation conidial progeny derived from stopper-B strains, suggest that this stopper phenotype too is determined by extrachromosomal factors.

Heterokaryotic Transmission of the Stopper-B Phenotype. The cytoplasmic nature of the genetic determinants of the stopper-B phenotype could be confirmed by the heterokaryon test. Strains with normal growth were found to grow in a

stop-start fashion after heterokaryotic association with stopper-B cultures. Heterokaryons between the ad var-1 stp-B strain of the B-growth tube and normally growing pan or nic cultures were prepared by superimposing conidia from the normal strains on 36-hour-old ad mycelium grown on slants of Vogel's adenine medium. This procedure yielded some heterokaryons with high proportions of ad nuclei. Heterokaryons which were prepared by the technique of Pittenger and Atwood (1955), or by simply superimposing conidia from the two homokaryotic components on minimal medium, invariably contained a very high proportion of nuclei from the normal component when the nuclear ratio was determined by the method of Atwood and Mukai (1955). The homokaryons which were recovered from such heterokaryons all grew normally in 500 mm tubes.

The ad stp-B homokaryons which were combined heterokaryotically with a normal pan culture were obtained as single conidia isolates from sections B80, B104, and B145. The growth behavior of adenine and pantothenate requiring single conidia isolates recovered from five heterokaryons is summarized in Table 3, which shows clearly that stopping segregated independently of the ad marked nucleus with which it was introduced into the heterokaryons. The appearance of stop-start growth in recovered pan homokaryons showed that the genetic determinants of the stopper-B phenotype are transmissible by heterokaryosis. Consequently, the

Table 3. Growth behavior of *ad* and *pan* homokaryons recovered from heterokaryons between *ad stp-B* single conidia isolates from the B-growth tube and a normally growing *pan* strain.

ad stp-B component of heterokaryon	Proportion of *ad* nuclei in heterokaryon	Recovered homokaryons tested in growth tubes		
		Nuclear marker	Total number	Number stopped
B80-3-221	0.89	ad	22	16
		pan	21	14
B104-3-13	0.64	ad	10	7
		pan	10	6
B104-4-1	0.98	ad	14	6
		pan	13	6
B104-4-18	0.89	ad	10	4
		pan	10	8
B145-3-26-8	0.69	ad	16	6
		pan	29	14
A-10 (Normal *ad* control)	0.81	ad	--	--
		pan	92	0

cytoplasmic nature of these factors, which was anticipated from the behavior of the phenotype during asexual and sexual reproduction, could be confirmed by transferring the phenotype from a stopper-B culture to a normal culture through intermediary heterokaryotic association of the two strains.

The stopper-B phenotype was also transferred from *pan stp-B* isolates to normally growing *nic* and *ad* strains. Of 27 *nic* homokaryons obtained from two heterokaryons between *pan stp-B* and a normal *nic* strain, 11 stopped in growth tubes. No stopping occurred in any one of 31 control *nic* homokaryons recovered from a heterokaryon with a normal *pan*

culture. Similarly, out of 9 ad homokaryons derived from a heterokaryon between a pan stp-B isolate and a normal ad, 5 grew in a stop-start manner. No stopping was observed in 20 ad control isolates.

Persistence of the Determinants of the Stopper-B Phenotype during Continuous Growth. The ad culture which was maintained in the B-growth tube grew as long as 519 days between consecutive stops. This posed the question whether two cessations of growth which were far apart one from another in time and distance, were the expression of a common, persistent factor, or if they were the consequence of independent mutational events. The expression of the stopper-B phenotype in single conidia isolates obtained at regular intervals from the continuously growing culture provided at least a qualitative means for distinguishing between the two alternatives. If consecutive cessations were caused by a persistent determinant, then stopping should have been observed in at least some of the conidial isolates from each intermittent sample, or perhaps even in mass conidial subcultures of each intermediary section, and the stopper phenotype should have remained comparable at all times. If independent mutations were responsible, then stopping would have been expected only in samples of isolates or subcultures which were taken from sections in the immediate vicinity of the stops, and the phenotype may or may not have been the same.

The longest interval of uninterrupted growth corresponded to the segment of the B-growth tube between sections B23 and B70, representing about 28,740 mm of continuous hyphal elongation. Single conidia isolates were obtained from subcultures of the following sections pertaining to this segment: B23, B25, B45, B50, B57, B63, B64, B66, B69, and B70. Stopping was found in some isolates from every sample, except the one corresponding to B40 (Table 4). No single conidia isolates were prepared from any one of the sections between B105 and B116, where the culture grew continuously for 108 days. However, 5 growth tubes were inoculated with masses of hyphae and conidia from every other section, i.e. B106, B108, B110, B112 and B114. There was a stop in at least one of the five tubes corresponding to each section.

After stopping once in section B116, the B-culture grew another 461 days without stopping, but stopping occurred in at least one of five growth tube subcultures of sections B118, B120, B122, B124, B130 and B142, and in none of the subcultures of sections B127 and B136. A fraction of the single conidia isolates from B145 and B150 also were found to stop (Table 4). In addition, it has been shown previously that the stopper-B growth behavior of one of the isolates from B145 could be transmitted to a normally growing strain by means of heterokaryosis (Table 3).

Table 4. Summary of the average growth rates for various sections of the B-growth tube, and of the total number of single conidia isolates, stopping isolates and the average growth rate of all isolates from each of these sections. Stopping in the main growth tube is indicated by S after the number of the section and a number indicating how often stopping occurred in that section.

Section		Single conidia isolates tested in growth tubes			
Number	Average rate (mm/day)	Total number	Number stopped	Proportion stopped	Average rate (mm/day)
*B23 S2	45	28	4	0.14	54
*B25	50	20	4	0.20	52
*B40	63	20	0	0.00	61
*B45	49	20	12	0.60	56
*B50	50	20	11	0.55	46
*B57	63	14	3	0.21	54
*B63	66	20	10	0.50	55
*B64	66	55	8	0.14	54
*B66	68	19	5	0.26	59
B69	63	20	9	0.45	49
B70 S2	66	15	13	0.87	46
B71 S1	67	15	15	1.00	47
B72 S2	68	20	11	0.55	60
B75 S2	65	12	7	0.58	49
B76	72	21	4	0.19	63
B78	64	20	6	0.30	64
B79	64	46	35	0.76	61
B80	64	13	12	0.92	57
B82	65	18	13	0.72	57
B84	63	20	3	0.15	51
B86 S1	66	13	0	0.00	60
B89 S1	61	14	1	0.07	60
B93	62	18	2	0.11	55
B96	60	17	0	0.00	55
B102	59	20	1	0.05	57
B103	64	10	1	0.10	67
B104 S2	64	60	3	0.05	63
B105 S2	58	39	2	0.05	61
B116 S1	54	10	0	0.00	53
B117	65	9	0	0.00	53
B118	62	9	0	0.00	52
B120	61	10	0	0.00	52
B145	66	87	6	0.07	49
B150	69	19	2	0.10	49

*The single colony isolates for the sections with an asterisk were prepared from subcultures which had been stored for some time at 4°. All other isolates were obtained from conidia which were extracted directly from the B-growth tube.

Table 4 provides a summary of the growth behavior of single conidia isolates obtained from a variety of sections of the B-growth tube. It can be seen from this table that the relative number of isolates which stopped in growth tubes apparently was not related to stopping of the parental ad culture during continuous growth. No definite explanation for this lack of correlation can be given at this time. It is possible that the events which took place in the growing mycelium of the parental culture were not reflected in the overall growth behavior of the conidial progeny. There is no reason to believe that the factors which caused stopping in the parental culture were substantially different from those which caused stopping in single conidia isolates or mass conidial subcultures. However, it is possible that the number of stopping isolates was not related to the proportion of stopper factors in the parental mycelium, either because conidia were formed only in mycelium which was two- or three-days-worth of growth behind the growing frontier, or because stopping in the isolates was influenced in some way by the metabolic events associated with the formation, germination and growth of conidia. The significance of these factors is evident from the fact that stopping appeared in single conidia isolates and mass conidial subcultures, even though the parental culture did not stop during long periods of growth on either side of the spots from which samples were taken. It is for these reasons

that the experimental evidence for the persistence of the extrachromosomal genetic determinants of the stopper-B phenotype is qualitative rather than quantitative.

Interactions between stp, stp-A, stp-B, and poky. Intergenic complementation has been used widely to establish non-allelism of nuclear genes. Pittenger (1956) observed that a heterokaryotic mixture of two cytoplasmic mutants, poky and mi-4, temporarily restored normal growth. It was hoped that differences between stp (McDougall and Pittenger, 1966), stp-A and stp-B could be detected by growing the three stopper mutants in different combinations with each other and with poky. The strains used to obtain heteroplasmons, or mixtures of different cytoplasms, were ad-4 al-2 stp-A, pan-1 al-1 stp-A, ad-4 al-2 stp-B, pan-1 al-1 stp-B, pan-1 al-1 stp and pan-1 al-1 poky. Heterokaryons were made by superimposing masses of conidia and hyphae from pan and ad strains with different cytoplasmic growth phenotypes. Each combination was grown in three growth tubes on Vogel's minimal medium, whereas the homokaryotic controls were grown on medium with the appropriate supplement.

Heterokaryons between stp-A and stp-B cultures were phenotypically stopper-A, at least with respect to growth, stopping, the formation and germination of conidia, and the type of colonies formed on sorbose medium. No definite relationship of dominance was obtained in combinations between stp and stp-B. Heterokaryons between these two

cytoplasmic mutants had phenotypic properties which could be interpreted as mixtures of the phenotypes of the two components. No interaction of any kind was found between stp and stp-A, but it must be stressed at this point that the two variants were phenotypically indistinguishable, except that the initial growth of stp controls was more vigorous than that of stp-A controls. None of the stopper mutants gave a well defined interaction with poky.

There is no evidence for complementation between any two of the above four cytoplasmic mutants of Neurospora. However, a possible difference between stp and stp-A appears in a more subtle kind of interaction; namely, dominance of stp-A over stp-B, but not of stp over stp-B. Together with slight differences in vigor during the first few days of growth in growth tubes, the dissimilarity in the interaction of stp-A and stp with stp-B is the only observation which suggests that the spontaneous mutation which determines stopper-A growth is different from the ultraviolet induced mutation which determines the stopper phenotype described by McDougall and Pittenger (1966).

Deficiency in Cytochrome c Oxidase Activity in Stopper-A and Stopper-B Mitochondria. Evidence favoring defective electron-transport systems in stp-A and stp-B cultures was obtained by determining cytochrome c oxidase and cytochrome c reductase activities in partially purified preparations of mitochondria. Cytochrome c oxidase activity was 20 to 36

times lower in preparations from the cytoplasmic mutants than in preparations from a normal control (Table 5). However, cytochrome c reductase activity was normal, or nearly normal, in mitochondria from both mutants. These observations suggest that mitochondrial defects are part of both cytoplasmically determined phenotypes. Careful comparisons of the kinetics of cytochrome c oxidase from mutant and normal strains remain yet to be done. Such studies should reveal if a lack of enzyme, or structural and/or configurational changes in the enzyme protein account for reduced activity.

Table 5. Specific activities of cytochrome c oxidase and cytochrome c reductase in partially purified preparations of mitochondria from stopper-A, stopper-B and normally growing cultures of Neurospora. The table gives two independent determinations for each strain. Column 4 gives the ratios of the numerical values of the oxidase and reductase activities.

Phenotype of the culture	Enzyme activities: Cytochrome c oxidase (min^{-1}/mg)	Cytochrome c reductase (units/mg)	Oxidase / Reductase
Stopper-A	0.40	1.78	0.22
	0.25	0.88	0.28
Stopper-B	0.44	1.04	0.42
	0.30	1.21	0.25
Normal control	13.90	1.64	8.47
	9.00	1.11	8.11

Genetic Basis of Some Changes in Growth Rate Observed during Continuous Hyphal Propagation

Several significant decreases and increases in growth rate were observed during prolonged hyphal growth of the ad-4 al-2 strain used for these studies (Figure 1). Attempts were made to determine the genetic changes which were responsible for the declines in sections A3 to A4, A16 to A17, B19 to B23, B40 to B46, and B151 to B157, and for the increases in sections B24 to B40, and B49 to B70. Since the environmental and nutritional conditions were kept as constant as possible during the extended period of growth, it appeared reasonable to assume that the changes in rate were due to intracellular alterations of the organism. Nevertheless, the involvement of heritable nuclear or cytoplasmic factors could be shown conclusively for only three decreases, and for none of the increases.

Decline in Rate in Sections A3 to A4. The A-culture grew about 138 mm/day prior to the decrease in rate to about 84 mm/day in section A5. Comparable rates are rarely observed in even the most vigorous strains of Neurospora. Such high rates were not observed subsequently when control subcultures of the ad strain, and subcultures and single conidia isolates from subcultures of sections A1 and A2 were grown in test tubes. In these cases the rates were comparable to the rate attained by the continuously growing culture at the end of the decline, i.e. between 80 mm/day

and 90 mm/day. McDougall (1963) used the same ad strain in a study on induction of extrachromosomal mutants by ultraviolet light, and reported its growth rate at approximately 80 mm/day. In spite of these observations, the high rate of growth of the ad culture during the first few days of continuous hyphal propagation was real, for it persisted over a distance of nearly 2100 mm. It appears that the factors which caused the initial vigor were not heritable; rather that they were either nutritional or environmental.

Decline in Sections A16 and A17. The probable involvement of cytoplasmic determinants in the decline in rate from 84 mm/day in section A15 to 24 mm/day in section A18 has been reported previously (McDougall and Pittenger, 1962; McDougall, 1963). McDougall (1963) observed that single conidia isolates from section A20 grew erratically, but some attained growth rates which were superior to that observed for the parent culture in the continuous growth tube.

It was of interest to determine how much of the variation in rate among single conidia isolates with a common origin should be attributed to nuclear heterogeneity. A subculture of section A19 was heterokaryotically combined with a normally growing pan strain, such that about 90 per cent of the nuclei of the heterokaryon were of the pan type. It was assumed that nuclear proportions could be considered indicative of the relative amounts of "normal" and "abnormal" cytoplasm in the heterocytosome. On this basis it was predicted that recovered ad homokaryons should be "cured" of

cytoplasmic factors for slow growth and their growth rates should be at about 80 mm/day, unless the heterogeneous rates of single conidia isolates were due to random assortment of nuclear factors during the abstriction of asexual spores.

The growth rates of 20 single conidia isolates from the subculture of section A19 ranged from 13 mm/day to 63 mm/day, with an overall average of 39 mm/day. Stopping of the stopper-A type was observed in 6 of these isolates. The rates of 18 ad homokaryons which were recovered from the disproportionate heterokaryon were between 69 mm/day and 86 mm/day, and averaged 81 mm/day. It was concluded that slow growth after the decline in sections A16 and A17 was determined by factors of extranuclear origin. The occurrence of stop-start growth among single conidia isolates from section A19 suggests that the decrease in rate may have been caused at least in part by the cytoplasmic changes which eventually lead to stop-start growth of the A-culture.

Decline in Rate in Sections B19 through B23. The decrease in growth rate in sections B19 through B23 of the B-growth tube coincided at least partly with the transition in phenotype of the ad culture from that of the ad-4 al-2 originally used to initiate continuous growth to one which was designated variant-1. Furthermore, a temporary cessation of growth was observed in B23 at the end of the decline. Since the prime objective for initiating the B-culture from the proximal end of section A16 was to investigate whether

or not the factor, or factors, responsible for the changes in A16 and subsequent sections were already present at the beginning of that section, it was of interest to determine if genetic factors were responsible for the decline, and to locate such factors either in the nucleus or in the cytoplasm.

The properties associated with the variant-1 phenotype--reduced formation of conidia, limited aerial growth, inability to function as a protoperithecial parent and lysis--were retained by the culture during more than three years of growth subsequent to section B23. This fact alone suggested that the phenotype was the consequence of an altered genotype. Since the transition to the variant-1 phenotype preceded stopping in section B23 by only a short time, and since the cytoplasmic determinants of the stopper-B phenotype were persistent during subsequent growth, it appeared possible that both stopping and the properties associated with the variant-1 phenotype were caused by one and the same genetic change. Furthermore, McDougall (1963) observed improved production of conidia in ad homokaryons from section B42 which were passed through a heterokaryon with a normal pan strain. This finding favored a cytoplasmic basis for limited formation of conidia, which is one of the characteristics of the variant-1 phenotype.

Later observations indicated that none of the properties associated with the variant-1 phenotype could be

modified substantially by passage of *ad var-1* strains through heterokaryons with normal cultures. Slight improvements, however, were noticed occasionally, particularly with respect to growth rate and the production of conidia. The results obtained from this type of somatic study suggested that reduced growth rate, limited aerial growth, sparse formation of conidia, the incapacity to form functional protoperithecia and lysis all were the consequence of mutations in one or more nuclear gene, and that cytoplasmic factors sometimes influenced the expression of the mutant genotype.

Nuclear determination of the variant-1 phenotype was confirmed by crossing a *74a* wild type culture as protoperithecial parent to *ad var-1* from section B82. A total of 29 complete unordered tetrads and 75 random spores from three crosses were analyzed. A simple visual examination of the complete tetrads revealed that four of the eight cultures from each ascus formed relatively few conidia. Using limited conidiation as a means of tentatively identifying the variant-1 phenotype, the ascospore progeny could be classified into four distinct classes: (1) nutritional wild type with wild type formation of conidia (*ad*$^+$ *var-1*$^+$); (2) nutritional wild type with reduced formation of conidia (*ad*$^+$ *var-1*); (3) adenine-requiring with wild type conidia formation (*ad var-1*$^+$); and (4) adenine-requiring with reduced formation of conidia (*ad var-1*).

All progeny were tested for the capacity to form functional protoperithecia, and the progeny from 6 complete tetrads were checked for the occurrence of lysis. The cultures with limited formation of conidia were incapable of functioning as protoperithecial parents and showed lytic spots in 12- to 20-day-old mycelium. All cultures with wild type formation of conidia functioned well as protoperithecial parents and did not lyse. The variant-1 phenotype segregated independently of mating type, requirement for adenine (ad-4), and albinism (al-2). The joint segregation of sparse formation of conidia, incapacity to differentiate functional protoperithecia, and lysis clearly indicates that these characteristics of the variant-1 phenotype are the consequence either of a mutation at a single chromosomal locus with pleiotropic effect, or of several mutations in a block of several very closely linked genes.

The effect of the var-1 genotype on growth rate was particularly apparent among the progeny obtained from the crosses between 74a and ad var-1. The average growth rates of single conidia isolates from both parents are given in Table 6, together with the average rates for each of the four types of sexual progeny. Some simple arithmetic showed that the characteristic rates of the four types of ascospore progeny were determined by two independently segregating loci, ad-4 and var-1. Both genes had depressive effects on rate, and their effects were additive.

Table 6. Average growth rates of single conidia isolates from cultures of wild-type 74a, ad-4 a1-2 var-1 from section B82, and ad-4 a1-2 var-1+; and of sexual progeny obtained from crosses between 74a and ad-4 a1-2 var-1. All rates were determined in 500 mm growth tubes.

	Number of progeny tested	Average growth rate (mm/day)
Single conidia isolates:		
74a	20	104 ± 4
ad var-1 from section B82	18	57 ± 6
ad var-1+ from sections A1 and A2	50	84 ± 2
Ascospore progeny from 74a x ad-4 a1-2 var-1 from section B82:		
ad+ var-1+	100	111 ± 14
ad var-1+	74	85 ± 6
ad+ var-1	54	69 ± 8
ad var-1	79	45 ± 10

Considering the 111 mm/day average rate of ad+ var-1 progeny as normal growth, then the average effect of the ad-4 was 85 mm/day - 111 mm/day = -26 mm/day. By the same reasoning, the average depression due to var-1 was 69 mm/day - 111 mm/day = -42 mm/day. The expected combined effect of the two loci was a reduction in rate of 68 mm/day, which is in close agreement with the 66 mm/day difference between the rates of ad+ var-1+ and ad var-1 progeny.

The 84 mm/day growth rate of the continuously growing ad culture in sections A4 through A15, and the average rate of single conidia isolates from subcultures of sections A1 and A2 (Table 5), are very close to the 85 mm/day average observed for ad var-1+ sexual progeny from above crosses.

This agreement further supports the belief that the initial vigor observed in A1 and A2 was due to factors other than hereditary determinants affecting growth rate.

There was no detectable difference between the variant-1 phenotype which was observed in single conidia isolates from a subculture of section B19 and that of the ad culture which grew in later sections. Among 46 random viable ascospores obtained from a cross between nic-1 al-2 88a and an ad-4 al-2 var-1A isolate from a subculture of B19, 21 were phenotypically variant, as expected for single-gene determination of a phenotype. A nic var-1 was selected from this cross and combined heterokaryotically with subcultures from sections B42, B62, B82, B120 and B140. Each combination was grown in three growth tubes. All heterokaryons showed sparse conidiation, were incapable of forming functional protoperithecia, and lysed. Their growth rates were between 57 mm/day and 70 mm/day, compared to rates between 56 mm/day and 69 mm/day for the ad components, and between 62 mm/day and 65 mm/day for the nic component. By virtue of lack of complementation in these heterokaryons, it can be assumed that the gene or genes determining the multiple properties associated with the variant-1 phenotype were the same in section B19 as in all subsequent sections of the B-growth tube.

On the basis of vigor alone it would appear that during continuous hyphal growth the ad var-1 component of a

heterokaryon between ad var-1$^+$ and ad var-1 should be at a selective disadvantage. However, evidence to the contrary could be obtained by inspecting single colony isolates from subcultures of various sections of the continuous growth tubes. The abundance of conidia formed on agar slants was used for visual distinction between var-1$^+$ and var-1 asexual progeny. The results are shown in Table 7.

Table 7. Relative numbers of var-1$^+$ and var-1 single conidia isolates obtained from subcultures of various sections of the A- and B-growth tubes.

Section	Total number of isolates	Phenotypes	
		var-1$^+$	var-1
A13	30	30	0
A14	30	30	0
A15	30	9	21
A19	38*	38	0
B16	29	13	16
B19	30	3	27
B20	30	8	22
B21	30	0	30
B23	30	0	30

*Includes 20 single colony isolates from a subculture and 18 adenine requiring, albino homokaryons reextracted from a heterokaryon of the same subculture with pan-1 al-1 23A.

Only isolates of the var-1$^+$ type were found in samples from sections A13 and A14, but both var-1$^+$ and var-1 progeny appeared in subcultures A15, B16, B19 and B20. Finally, only var-1 isolates were obtained from conidia of subcultures from B21 and B23. The relative proportions of the two types of asexual derivatives probably are not indicative of the

actual proportions of the corresponding nuclei in the mycelium at the time of growth. It has been observed repeatedly in this laboratory that subculturing of Neurospora heterokaryons may result in drastic changes in the relative proportion of the component nuclei. Whatever the effect of subculturing may have been in this case, it is clear that var-1 nuclei were present in the mycelium of the continuously growing culture long before the variant-1 phenotype became established. The displacement of var-1$^+$ nuclei by var-1 nuclei probably was a gradual process, though the observations do not exclude abrupt changes and fluctuations in nuclear ratio.

It is interesting to notice that the displacement of var-1$^+$ nuclei was complete in the B-growth tube, but never materialized in the A-growth tube. The reason for this difference is not clear. There is a strong possibility that selection at the nuclear level was influenced by the cytoplasmic changes which occurred at the same time.

Decline in Rate in Sections B151 through B157. That a gradual accumulation of mutant nuclei can occur during vegetative growth could be established conclusively by following the changes in nuclear composition of the continuously growing culture which took place during hyphal elongation prior to and during the decline in sections B151 through B157. A number of very small colonies with a peculiar morphology were observed in a plating of conidia

taken directly from a sampling port of B145. One or two hyphae extended from the conidium, and then branched extensively to give rise to a fan-like, single layer of growth on the surface of the solidified sorbose medium. When these colonies were transferred into growth tubes, the resulting cultures grew extremely slowly, at best 7.0 mm/day. The mycelium had the appearance of a flat thin rope with small lateral branches strung along the middle of the agar surface of the tube. There was no aerial growth and conidia were lacking completely. The relatedness of this new mutant to ad-4 al-2 var-1 was established by the fact that the mutant required adenine for growth and was heterokaryon positive with pan-1 al-1 23A.

The new phenotype was called variant-2, and the corresponding genotype was designated var-2. This phenotype could not be transferred to normal pan strains by means of heterokaryosis, nor was it reversed in ad homokaryons after heterokaryotic association with normal cultures. However, it was recovered in the progeny from a cross in which 74a was the protoperithecial, and ad var-2 the conidial parent. Among 14 viable spores recovered from this extremely infertile cross, 8 gave rise to cultures which generated an abundance of conidia, 2 formed only limited amounts of conidia as is typical of the variant-1 cultures, and 4 cultures had the characteristics described for variant-2 cultures. The appearance of both phenotypes, variant-1 and

variant-2, among the sexual progeny indicates that the corresponding genes are located in different segments of the genome. It is probable that *var-1 var-2* double mutants cannot be distinguished from *var-2* single mutants, except by genetic tests.

The proportion of variant-2 colonies increased gradually from 4 per cent in section B145 to nearly 18 per cent in B150 (Table 8). It remained at this level during almost two months of growth, until a rather sudden increase to 25 per cent occurred in B157. Even though there was no significant increase in variant-2 homokaryons, the growth rate of the culture declined steadily from 69 mm/day in B151 to 46 mm/day in B157 (Figure 1). There was no further decrease in rate until continuous growth was terminated in B160, but the culture turned aconidial at the end of B157, such that the assessment of further nuclear changes became virtually impossible.

Table 8. Proportions of *var-2* colonies in platings of conidia extracted directly from sections between B143 and B157, inclusive.

Section	Total number of colonies	Number of var-2 colonies	Per cent var-2 colonies
143	236	0	0
145	99	4	4.0
146	201	11	5.5
147	216	16	7.4
149	221	31	14.1
150	146	26	17.8
152	198	35	17.7
154	204	31	15.2
156	185	32	17.3
157	207	52	25.1

The variant-2 phenotype was suppressed in heterokaryons with var-1 and var-2 nuclei. Consequently, the percentages of variant-2 colonies given in column 4 of Table 8 represent the proportions of homokaryotic var-2 conidia present in the samples. The actual proportion of var-2 nuclei in the growing mycelium probably was several times higher, since in Neurospora heterokaryons the component nuclei assort independently during the abstriction of the multinucleate conidia (Atwood and Pittenger, 1955). Whatever the actual proportions of var-2 nuclei were, the increases in the proportion of variant-2 colonies during growth, together with the morphological change of the continuously growing ad culture from conidial to aconidial in section B157, clearly indicate that mutant nuclei can accumulate gradually during vegetative growth, even though the resulting phenotypic changes appear to be non-adaptive.

Changes in Growth Rate between Sections B23 and B70. Figure 1 shows two gradual increases and one decrease in growth rate during growth of the ad culture between sections B23 and B70. A specific cause explaining any one of these changes could not be pinpointed. Both nuclear and extranuclear factors which could have affected the growth of the culture were known to have been present in the mycelium during growth in this segment of the tube. For example, the cytoplasmic determinants of the stopper-B phenotype were shown previously to have persisted throughout this period

of uninterrupted growth, and minor improvements in the growth rate and production of conidia of ad homokaryons from this segment was noticed occasionally after passage through a heterokaryon with a normal strain. However, a number of isolates which grew uniformly at a rate of 38 mm/day were obtained from subcultures of sections B23, B25 and B45, where the rate of the parental culture reached low points. The growth rate of these isolates was not altered after heterokaryosis with a fast growing pan culture, suggesting that it was determined by nuclear factors. Consequently, the changes in growth rate between B23 and B70 could have been the consequence of changes in a population of heterogeneous nuclei, as well as the expression of alterations in the composition of the cytoplasm.

DISCUSSION AND CONCLUSIONS

The main objective for maintaining a culture of Neurospora by long term growth was to investigate the usefulness of prolonged hyphal elongation for the detection and isolation of extrachromosomal mutants. Two cytoplasmic variants, stp-A and stp-B, were obtained from the ad-4 al-2A culture. The characteristics of these mutants--heterokaryotic transmission of the phenotypes, failure to be transmitted in sexual crosses, vegetative persistence, and segregation in asexual spores--are consistent with the interpretation that the genetic determinants of both stopper phenotypes are of

cytoplasmic origin. Both mutants were detected within the first year of continuous growth. The determinants of stop-start growth persisted in the mycelium of the cultures until hyphal elongation was discontinued three to four years after stopping was noted for the first time in the A- and B- cultures. These observations provide experimental evidence for the occurrence of detectable cytoplasmic mutations during prolonged growth. The early occurrence of both mutants indicates that excessively long periods of growth are not required before mutants can be obtained. Therefore, the method is feasible as a laboratory technique. The persistence of the extrachromosomal determinants of the stopper-A and stopper-B phenotypes suggests that timing may not be very critical for the isolation of new mutants, at least not for some types.

The full potential of vegetatively propagated fungi as sources of cytoplasmic mutants hardly can be realized from the isolation of two variants from diverging growth lines of the same culture. At present, the difficulty in the isolation is one of recognition. The system used in this investigation was restricted to a certain type of mutant by two conditions: (1) the mutation which occurred in one of many sets of genetic information contained in the cytoplasm of the coenocytic mycelium was accumulated eventually, i.e. it was suppressive, and (2) the accumulation of mutant sets of genetic information resulted in a noticeable modification in

the phenotype of growth and/or reproduction. The only technique which has been used successfully for selecting induced cytoplasmic mutants of Neurospora involves the isolation of asexual progeny with aberrant growth behavior (Srb, 1963; McDougall and Pittenger, 1966). Since abnormally growing cultures often have defective reproductive systems, and may be difficult to maintain, the distinction between chromosomal and extrachromosomal inheritance of phenotypic traits can become a very tedious task.

Although stp-A and stp-B both are characterized by stop-start growth, several phenotypic differences support the notion that they are different mutants. The initial growth of stp-A cultures is subnormal, the periods of complete absence of growth are frequent and long, and intermittent active hyphal elongation lasts only a few days at a time. Cultures of the stp-B variant grow normally during the first few days after innoculation, stops are infrequent and last only up to four days, and very long periods of intermittent growth can occur during prolonged hyphal propagation. Growth invariably resumes some distance behind stopped hyphal tips of stp-A mycelium, but resumption of growth appears to occur at, or very near to, the stopped frontier of stp-B cultures. The formation of conidia by stp-A cultures is absent in growth tubes, but subcultures which are grown on agar slants occasionally produce abundant conidia. Conidiation is reduced in stp-B cultures, but

approximately equal amounts of asexual spores are produced in growth tubes and on slants. Only about 1.0 per cent of stp-A conidia germinate, and growth after germination rarely proceeds beyond the germling stage, but stp-B conidia germinate and grow normally. The characteristics of stp-A are very similar to those of the UV-induced stp variant described by McDougall and Pittenger (1966). Nevertheless, stp-A was dominant over stp-B in heteroplasmons, whereas mixtures of stp and stp-B gave rise to an intermediary phenotype with characteristics from both components. From these observations can be concluded that stp, stp-A and stp-B involve three different extrachromosomal mutations.

Since the stopper phenotypes are transmissible by heterokaryosis, they are not caused by the loss of some cytoplasmic element. A loss of this type also would be inconsistent with the persistence of the phenotypes during years of vegetative growth. Rather, the observations point to a change or loss of some function of a cytoplasmic component whose multiplication and inheritance is at least partially independent from chromosomal genetic information. Depressed cytochrome c oxidase activity in crude preparations of mitochondria from stp-A and stp-B mycelium indicates that the mitochondrion is the cytoplasmic component in question.

Although the concepts of cytoplasmic heredity have been evolving for many years, the only well known class of extrachromosomal mutations affecting mitochondria are found

in the respiratory deficient variants of yeast and Neurospora (Wilkie, 1964; Jinks, 1964). Usually several components of the electron transport system are found either in excess or in deficit in these mutants (Ephrussi, 1953; Mitchell, Mitchell and Tissieres, 1953; Diacumakos, Garnjobst and Tatum, 1965). Furthermore, mitochondria from a number of the variants are deficient with respect to succinic acid oxidase activity (Ephrussi, 1953; Haskins, Tissieres, Mitchell and Mitchell, 1953), and show altered malate dehydrogenase kinetics (Munkres and Woodward, 1966).

Certain cytoplasmic mutations affecting mitochondria probably are expressed pleiotropically because they may affect the assembly and overall organization of enzyme systems involved in vectorial catalysis, such as the electron transport chain, rather than the primary structure of the enzyme proteins. For example, poky is deficient with respect to cytochrome a, but has an excess of cytochrome c (Tissieres, Mitchell and Haskins, 1953). Nevertheless, wild type and poky cytochrome c have identical electrophoretic mobility, amino acid composition, and tryptic peptide maps (Heller and Smith, 1966). The mitochondrial structural protein of two extrachromosomal mutants of Neurospora, poky and mi-3, apparently differ from mitochondrial structural protein of wild-type strains by single amino acid substitutions (Woodward and Munkres, 1966). It is believed that the function of this protein is structural

rather than catalytic, and that it is involved in the binding of a number of enzymes associated with the mitochondrion. Alterations in this protein, therefore, not only could alter the activity of a single enzyme, but also could disturb entire pathways by altering the spatial relationship between two or more enzymes participating in vectorial catalysis. In the case of stp-A and stp-B, kinetic studies of mitochondrion-bound and dissociated cytochrome c oxidase might reveal whether these mutations affect the primary structure of the enzyme, or if reduced activity is simply the expression of a change in another component of the mitochondrion. The mitochondrial structural protein of stp appears to be "wild type" (Woodward and Munkres, 1966), but there is some evidence for an excess of cytochrome c (McDougall and Pittenger, 1966).

The most prominent characteristic of the two cytoplasmic mutants obtained from the continuously growing culture is their stop-start growth pattern. As mentioned previously, this type of growth is also characteristic of the UV induced stp variant (McDougall and Pittenger, 1966), and furthermore has been noticed in poky and mi-4 (Pittenger, 1956), SG (Srb, 1963), and abn-1 and abn-2 (Garnjobst, Wilson and Tatum, 1965). Though the physiological basis of stopping is unknown, there is evidence that in mi-4 (Pittenger, unpublished), stp (McDougall and Pittenger, 1966) and stp-A it is related to a high proportion of an

abnormal cytoplasmic component. Depending on high or low input of normal cytoplasm during heterokaryon formation, normal or mutant initial growth is observed in the heteroplasmons, and in single conidia isolates obtained from these heterokaryons.

Most of the cytoplasmic mutants which have been studied in fungi are suppressive (Jinks, 1964). This observation is not unexpected, because routine methods are not suited for the detection of non-suppressive mutations. Heteroplasmons involving wild-type and mutant cytoplasms usually result in a gradual elimination of the normal component. Jinks (1964) favors the interpretation that a superior rate of division of the abnormal cytoplasmic component results in the breakdown of the heteroplasmon. This mechanism could account for the appearance of the stopper phenotypes during prolonged hyphal growth, as well as for stopping. The overproduction of abnormal mitochondria, for example, could lead to a gradual displacement of normal organelles which are essential for the generation of certain metabolites required for normal growth. In this sense, the depletion of one or more essential metabolites, rather than the accumulation of abnormal mitochondria would be the immediate cause of stopping. It appears reasonable that such cellular processes would be expressed phenotypically as a gradual decrease in growth rate ending with a total cessation of hyphal elongation.

Although superior replication rates of abnormal cytoplasmic factors may satisfactorily explain suppressiveness and stopping, this theory may be a gross oversimplification of the actual processes involved in the case of stp-A and stp-B. Any mechanism which is proposed to explain stopping must also account for the resumption of growth. Resumption per se can be disposed of by arguing that there are small areas in the mycelium with sufficiently high proportions of normal factors such that new growth can be supported. Such areas, perhaps, should be found preferentially some distance behind the stopped frontier. Indeed, in stp-A cultures growth does resume well behind the stopped frontier, but there is a considerable delay before new growth appears. As a consequence, concentration of normal factors alone cannot be involved in this case, because there is little reason to believe that a favorable concentration is attained two weeks, or even three months, after the cessation of growth, if it did not exist at the time when growth stopped. Rather, delayed resumtion of growth suggests the participation of some kind of regulatory mechanism. The release from a repressor control, for instance, could be a prerequisite for the reinitiation of growth. Furthermore, in stp-B cultures growth always resumes in the immediate vicinity of the stopped frontier, suggesting that in this mutant the resumption is not dependent upon concentration of normal factors in the same manner as in stp-A

Another complication yet arises from the fact that the recurrence of stopping in the continuously growing ad stp-B culture was unpredictable. The B-culture grew for periods as short as a few days and as long as 17 months between consecutive stops. If stopping was caused by an increasing proportion of abnormal cytoplasmic elements which divide more rapidly than their normal counterparts, then long periods of uninterrupted vegetative growth must have been the consequence of the establishment of an equilibrium between the production rates of normal and abnormal factors. Complete elimination of the genetic determinants for stopping is ruled out by the observation that stopping occurred in single colony isolates obtained from various points of the growth tube between consecutive stops. An equilibrium in the division rates would suggest the involvement of still another regulatory mechanism. Changes in the internal environment of the mycelium may have been responsible for temporary ineffectiveness of the regulatory mechanism, such that stopping appeared in series between long periods of growth. Similar upsets may have appeared frequently when the organism reproduced asexually, since stopping occurred in a variable number of single conidia isolates even though the parental culture grew normally.

A modified version of the theory of differential division rates of normal and abnormal cytoplasmic elements has been proposed by McDougall and Pittenger (1966) as a

possible explanation for stop-start growth. The accumulation of a selfintoxicating substance, rather than the depletion of essential metabolites, could cause the cessation of growth when the proportion of abnormal factors in the mycelium reaches a certain level. Subsequent elimination of this substance, by diffusion or decay, would permit the resumption of growth. Elimination by diffusion apparently is not the case in stp-A and stp-B cultures, since the frequency and duration of stops under conditions which facilitate diffusion is the same as under conditions which are adverse to diffusion. If a non-diffusible, unstable substance is produced at all, then its properties or metabolism could not be the same in stp-A as in stp-B, since the types of stop-start growth associated with these two mutations are very different, even in the presence of the same nuclear background. It should be pointed out that an intoxicating substance could be a component of a regulatory system, or some type of analog of such a component.

In summary, observations with stp-A and stp-B cultures provide some evidence favoring that the suppressiveness and the stop-start growth of these two cytoplasmic mutants of Neurospora may not be explained simply in terms of different rates of multiplication of two alternate forms of some cellular organelle. Diacumakos, Garnjobst and Tatum (1965) concluded that the simple coexistence and independent functioning of normal and abn-1 mitochondria in the same

cytoplasm appeared to be unlikely, because the cytochrome spectrum of heteroplasmons between abn-1 and normal strains revealed amounts of cytochrome c lower, and amounts of cytochrome a and b higher than expected for simple mixtures of abnormal and normal mitochondria. On the basis of this observation, the above authors suggest that complex regulatory interactions, or transformation of normal mitochondria into abnormal ones by substances liberated by degenerating abn-1 mitochondria may be responsible for the unexpected cytochrome spectrum. Transformation of normal into abnormal organelles appears to be of particular interest, because this process must be considered as an alternative to differential division rates for the explanation of suppressiveness, and possibly as the process responsible for stopping. Unambiguous experimental evidence for the occurrence of either differential division rates or transformation has not been provided yet. However, it appears that certain cytoplasmic mutants, particularly the three stopper variants and poky, have properties which may be useful for the solution of this problem.

The naturally occurring genetic changes which appeared during long term vegetative propagation of Neurospora resulted mainly in the degeneration of growth performance and reproductive capacity of the ad-4 al-2A culture used for these studies. In this respect, the observations parallel those made by Jinks (1959) with Aspergillus; except that in

Neurospora the phenotypic changes could be ascribed to permanent genetic alterations at the extrachromosomal and chromosomal level, rather than to reversible cytoplasmic changes resulting from readjustments in the equilibrium of normal cytoplasmic constituents. There was also no indication that in Neurospora an irreversible cessation of growth is the final condition of the degenerative process associated with prolonged vegetative propagation, as observed for Aspergillus (Jinks, 1959) and Podospora (Marcou, 1961). The extrachromosomal genetic determinants of the stopper-A and -B phenotypes, and death of stopped hyphal tips suggest the possibility that stp-A and stp-B, and "vegetative death" in Aspergillus (Jinks, 1959) and "senescence" in Podospora (Marcou, 1961) are related. Occasional resumptions of growth have been observed in dying cultures of Aspergillus, but these recoveries are short lived (Jinks, 1959), and apparently do not occur repeatedly in the same culture. This is not the case with Neurospora stopper mutants, where temporary cessations are the rule rather than the exception, and recoveries may be long lived. Consequently, if there is any relationship between the stopper phenotypes of Neurospora and irreversible cessation of growth in Aspergillus and Podospora, then Neurospora has biological properties which permit the organism to survive a cytoplasmic change which is lethal in some other fungi. These properties may be vigorous cytoplasmic streaming and rapid growth, both of which are

distinctive characteristics of Neurospora.

Nuclear participation in changes in phenotype arising during prolonged growth was found to be significant in Neurospora. Gradual displacements of one type of nucleus by another took place in two different portions of the B-growth tube. In sections B16 through B22 var-1^{+} nuclei were gradually eliminated by var-1 nuclei. In sections B145 to B160, the gradual replacement of var-1 by var-2 nuclei was not complete, but progressed to the extent that the culture began to take on properties which were characteristic of var-2 homokaryons. Both displacements appeared to be very slow processes which extended over several months of growth. The resulting decreases in growth rate, however, occurred much more rapidly and at stages where the type of nucleus which was on the increase had reached high proportions in the population of nuclei present in the mycelium.

Pittenger and Atwood (1955) reported that in heterokaryons involving biochemical mutants the proportions of the component nuclei are very stable between ends of 300 mm growth tubes. Obviously, changes in proportion of the type observed during continuous growth could not be detected in short growth tubes. More recently, Pittenger and Grindle (unpublished) have studied the effects of long term growth on nuclear ratios in heterokaryons between complementary biochemical mutants. Gradual changes in nuclear proportions similar to those described above were observed, among

other things, but in these systems the complete displacement of one type of nucleus by another apparently was prevented by physiological interdependence due to nutritional requirements.

Similar to the accumulation of cytoplasmic factors, the nuclear changes observed in the continuously growing ad cultures may be explained on the basis of small differences in division rate, at least in theory. Furthermore, there are indications that this explanation by itself may be an oversimplification of the processes which actually took place during growth, an observation which also was made for the cytoplasmic changes. For example, the proportion of var-2 homokaryons increased from 4 per cent to 17 per cent in the length of six growth tubes, from B145 to B150, but no further increase occurred in the six sections which came after B150. A continuous increase would be predicted if division rates alone were decisive. Though there appears to be some analogy between the accumulation of mutant nuclei and the accumulation of mutant cytoplasmic factors, the information on the cellular mechanisms which are responsible for the accumulations is yet too limited to ascertain that basically the same processes are involved at the cytoplasmic and the nuclear level.

Interestingly, in both cases of nuclear displacement reported in this dissertation, selection was against the type of nucleus which was responsible for more vigorous

growth. Davis (1966) mentions evidence supporting the opinion that individual hyphae of a growing culture can respond to local differences in nuclear composition. Such response apparently was absent during the two accumulations of nuclei determining slow growth, because hyphae with high proportions of nuclei determining fast growth should have outgrown other hyphal tips, thus providing a selective pressure favoring nuclei for fast growth. In actuality, the decreases in rate occurred rather gradually and after the mutant nuclei reached high proportions, as expected for a response of the growing culture as a whole to the nuclear composition of the mycelium. Apparently cytoplasmic streaming and hyphal anastomoses are very effective for the distribution of hyphal contents throughout the growing frontier, such that local differences may be short lived and of little significance for the selection of nuclei.

Atwood, Schneider and Ryan (1951) concluded from long term population studies with bacteria that classes of mutations arise which control small differences of adaptive value. The mutant types replace the existing population during growth. It is difficult to imagine how the var-1 and var-2 mutations determined differences of adaptive value. Furthermore, it is doubtful whether the replacement of an existing population of nuclei by newly arising mutant nuclei within a coenocytic mycelium is comparable to selection in a population of unicellular organisms. It appears that in

Neurospora the accumulation of a nucleus with a mutation which is not necessarily of selective advantage can occur simply by virtue of the fact that the wild-type nuclei in the population insure normal metabolic processes. However, the expression of individual genomes is critical for the survival of individual cells in a population of unicellular organisms with one or two nuclei per cell, since the interaction between nuclei of different cells is negligible, unless there is rather active sexual recombination. For example, the superior division rate of a nucleus with a deleterious mutation may lead to a significant increase in the proportion of that nucleus in the population of nuclei found in the coenocytic mycelium of a growing culture of Neurospora, but the superior division rate of a genome with a deleterious mutation would be ineffectual in a population of bacteria, because mutant organisms would be eliminated. In conclusion, mutant nuclei, which may or may not be of adaptive value, can be accumulated in growing cultures of Neurospora, at times to the complete exclusion of existing nuclei.

One of the phenotypic characteristics of var-1, "lysis," deserves brief consideration. Catcheside (1959) reported that Lewis has unpublished observations of lysis in the fungus Coprinus lagopus, which is controlled by a nuclear gene, as is the case in Neurospora. Recent observations with Neurospora strains which are unrelated to the al-4 al-2

culture used for continuous growth, indicate that lytic deterioration of ageing mycelium may be relatively common in this fungus. It occurs in several degrees of severity, and in the most pronounced cases lytic spots appeared in three- to five-day-old mycelium. The strains which show lysis in less than three-week-old mycelium appear to be incapable of functioning as protoperithecial parents in crosses, and also form few or no conidia. Lysis appears to be particularly severe in strains with rhythmic growth, the so called "clock mutants." Indications are that all types of lysis in Neurospora are determined by mutations at the chromosomal level. Some of the mutants have arisen spontaneously, but others were obtained after treatments with N-methyl-N'-nitro-N-nitrosoguanidine. These mutants appear to be of singular interest, because of the possibility that they are cell membrane or cell wall mutants, and thus may prove useful for studies of these structures.

SUMMARY

An ad-4 al-2A strain of Neurospora crassa was grown continuously for more than four years to determine if extra-nuclear and nuclear genetic changes were responsible for some of the somatic variation which appears during prolonged hyphal propagation. A branch culture (B-culture) was derived from the main culture (A-culture) after three months of growth, and subsequently both cultures were maintained

in parallel.

Although there were several significant changes in the growth rate of the strain, the most unusual feature was a number of temporary cessations of growth which appeared in both cultures before the first year of vegetative propagation was completed. The stop-start growth of the A- and B- cultures was found to be characteristic of two different cytoplasmic mutants, stp-A and stp-B, respectively.

The stp-A variant had erratic initial growth, long stops, short periods of growth, one per cent germination of conidia, and colonies ranging from germlings to small macrocolonies. The stp-B variant showed normal initial growth, short stops, long and short periods of growth, and normal germination and growth of conidia. Both mutants formed few conidia and no functional protoperithecia. Hyphal tips from stopped cultures of either variant did not grow when transferred to fresh medium, nor did they form viable heterokaryons with normal strains. The stopper phenotypes did not segregate at meiosis, but could be transmitted to normal strains through heterokaryosis. Mitochondria from both mutants were deficient in cytochrome c oxidase activity. The two spontaneous cytoplasmic mutants appear to be different from the UV-induced stp variant.

Though a permanent cessation of growth was not observed in Neurospora, the characteristics of stp-A and stp-B suggest that these variants may be related to "vegetative

death" in Aspergillus and "senescence" in Podospora.

Although the physiological basis of stopping is unknown, several possible explanations have been considered. An overproduction of mutant cytoplasmic factors possibly results in a displacement of their normal counterpart. This displacement could produce either a deficiency of some essential metabolite, or in the synthesis of intolerable amounts of the endproduct of the mutated gene, or genes. Either process would result in a gradual decrease in growth rate and stopping. The variability of the duration of the periods of active and inactive growth, and the differences between the stopper phenotypes suggest that control mechanisms are involved in stop-start growth, in addition to simple changes in the proportion of normal and mutant cytoplasmic elements.

The most pronounced nuclear effects observed during long term growth of Neurospora were two gradual accumulations of mutant nuclei in the B-culture. A complete displacement of the original ad-4 al-2 nuclei by nuclei with a mutation which caused a reduction in rate, sparse conidiation, female sterility and lysis occurred during the first accumulation. The new genotype of the culture was called ad-4 al-2 var-1. The mutation responsible for the multiple alterations in phenotype appeared to involve a single gene, or a block of closely linked genes. The second accumulation resulted in a gradual, but not complete substitution of ad-4 al-2

var-1 nuclei by *ad-4 al-2 var-2* nuclei. Homokaryons of the *var-2* type grew less than 7 mm/day, were aconidial and had a ropy appearance.

Differences in nuclear division rates and nuclear selection in a growing coenocytic organism have been considered in an attempt to explain the accumulation of a mutant genome during hyphal propagation.

Some of the changes in growth rate A- and B-cultures could be attributed to specific changes in the cytoplasmic or nuclear genetic composition of the mycelium, whereas others involved nutritional or environmental factors, and subtle cytoplasmic and/or nuclear alterations which could not be identified with certainty.

All cytoplasmic and nuclear mutations which were amenable to genetic analysis were non-adaptive with respect to growth vigor. Furthermore, these mutations were suppressive in the sense that mutant factors were accumulated during growth until the mutant genotype was expressed phenotypically.

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NATURALLY OCCURRING CYTOPLASMIC AND NUCLEAR CHANGES IN CONTINUOUSLY GROWING CULTURES OF *NEUROSPORA CRASSA*

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ABSTRACT

The special significance of mitochondria in cellular heredity has been recognized with the discovery of DNA in these organelles. However, additional mutants are needed to learn more of the role of extrachromosomal genetic material in the physiology of the cell. Most cytoplasmic mutants in fungi are suppressive in that the proportion of mutant determinants in heteroplasmons increases during growth. Since both time and growth are decisive in this process, continuous hyphal propagation should provide the conditions favoring the detection of spontaneous suppressive cytoplasmic variants.

An ad-4 al-2A strain of Neurospora crassa was grown continuously for over four years to determine the genetic basis of somatic variations arising during prolonged hyphal elongation. A branch culture (B-culture) was derived from the main culture (A-culture) when a decline in growth rate appeared after three months of growth, and subsequently both cultures were maintained in parallel. Although there were several significant changes in the growth rate of the strain, the most unusual feature was a number of temporary cessations of growth which appeared repeatedly in both cultures. This stop-start growth of the A- and B-cultures was found to be characteristic of two cytoplasmic mutants, stp-A and stp-B, respectively. The stp-A variant had erratic initial growth, long stops, short periods of growth, one

per cent germination of conidia, and colonies ranging from germlings to small macrocolonies. The stp-B variant showed normal initial growth, short stops, long and short periods of growth, and normal germination and growth of conidia. Both mutants formed few conidia and were female sterile. Hyphal tips from stopped cultures of either variant did not grow when transferred to fresh medium, nor did they form viable heterokaryons with normal strains. The stopper phenotypes did not segregate at meiosis, but were transmitted to normal strains through heterokaryosis. Mitochondria from both mutants were deficient in cytochrome oxidase activity.

Stop-start growth appeared to involve not only changes in the proportion of normal and mutant cytoplasmic factors, but also control mechanisms affecting the length of the periods of active and inactive hyphal elongation. Although a permanent cessation of growth was not observed in Neurospora, the characteristics of stp-A and stp-B suggest that these variants are related to "vegetative death" in Aspergillus, and "senescence in Podospora and other fungi.

The most prominent nuclear effects encountered during prolonged growth of Neurospora were two gradual accumulations of mutant nuclei in the B-culture. During the first accumulation, the original ad-4 al-2 nuclei were displaced entirely by ad-4 al-2 var-1 nuclei, determining reduced growth rate, sparse conidiation, female sterility and lysis of aged mycelium. The second accumulation resulted in a

gradual, but not complete, substitution of ad-4 al-2 var-1 nuclei by ad-4 al-2 var-2 nuclei. Homokaryons of the var-2 type grew very slowly, were aconidial, and had a ropy appearance. Differences in nuclear division rates and nuclear selection in a growing coenocytic organism possibly explain the increase in the proportion of mutant genomes during growth.

Some of the growth rate changes in the two cultures could be attributed to specific alterations in the cytoplasmic or nuclear genetic composition of the mycelium, but others involved nutritional or environmental factors, and subtle genetic changes which could not be identified with certainty. Those mutations which were amenable to genetic analysis were non-adaptive with respect to growth vigor, and suppressive in the sense that mutant factors accumulated during growth until the mutant genotype was expressed phenotypically.